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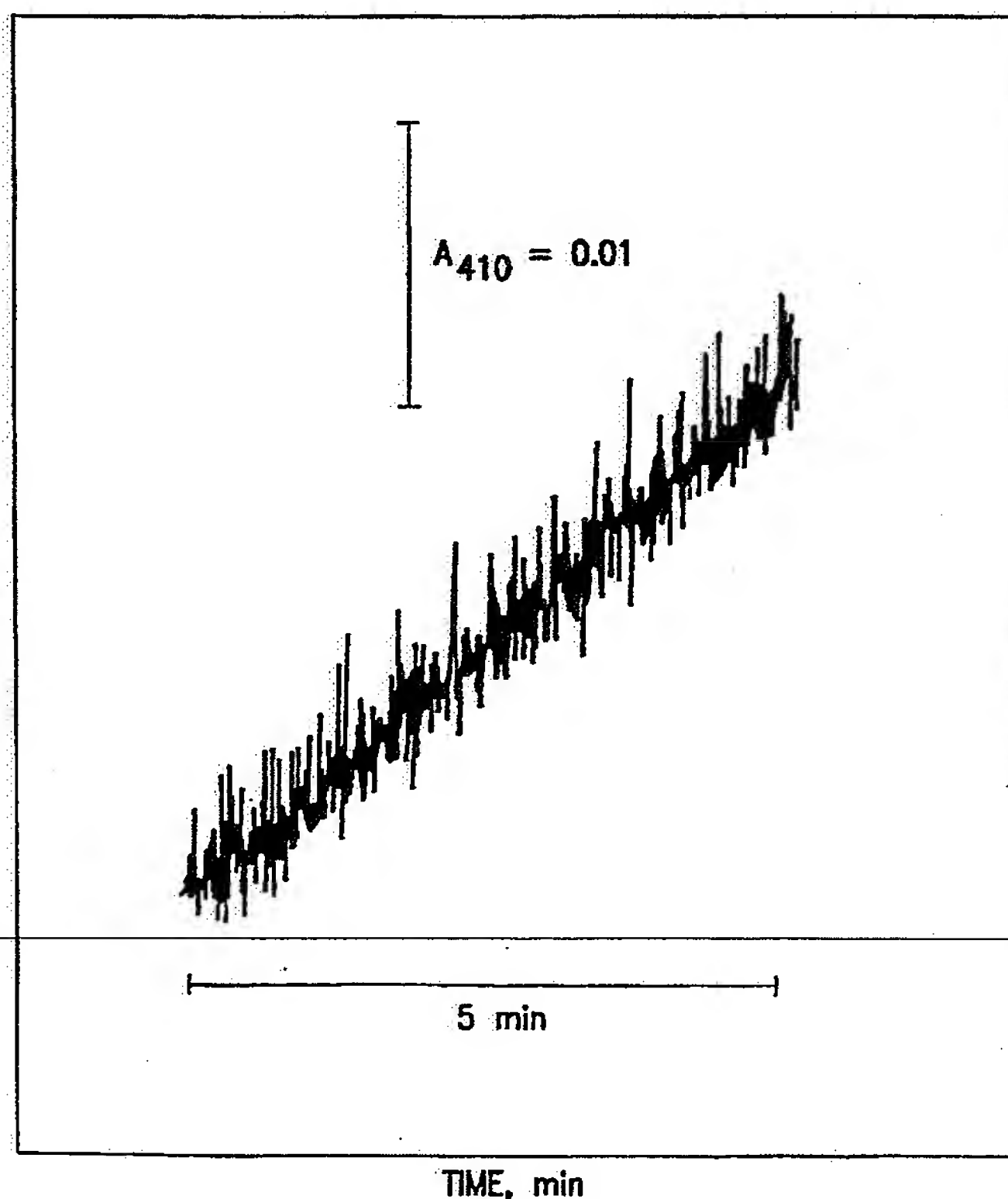
US

(71) Applicant: PURDUE RESEARCH FOUNDATION [US/US];  
1063 Hovde Hall, West Lafayette, IN 47907-1063 (US).(72) Inventors: MORRE, Dorothy, M.; 1112 Cherry Lane, West  
Lafayette, IN 47906 (US). MORRE, D., James; 1112 Cherry  
Lane, West Lafayette, IN 47906 (US).(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds LLP,  
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THEREOF

## (57) Abstract

The invention described herein encompasses methods of preventing or treating disorders caused by oxidative damage by an aging-specific isoform of NADH oxidase (AR-NOX). The invention encompasses methods of assaying, screening, and identifying agents that inhibit AR-NOX, as well as methods using ubiquinone to inhibit the ability of AR-NOX to generate reactive oxygen species. These agents may be formulated into pharmaceutical compositions in the prevention and treatment of disorders caused by oxidative damage.

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## METHODS FOR IDENTIFYING AGENTS THAT INHIBIT SERUM AGING FACTORS AND USES AND COMPOSITIONS THEREOF

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5                   This application claims benefit of U.S. provisional application serial No. 60/126,894 filed March 30, 1999.

### 1. INTRODUCTION

10                   The present invention relates to methods for the prevention or treatment of disorders and complications of disorders resulting from cell damage caused by an aging-related isoform of NADH oxidase (AR-NOX). The invention comprises assays for screening for agents that bind AR-NOX and inhibit the ability of AR-NOX to generate reactive oxygen species as well as methods of using ubiquinone to inhibit the ability of AR-NOX to generate reactive oxygen species. The invention also encompasses using the  
15                   therapeutic compounds detected in the screening assays of the invention in a pharmaceutically acceptable carrier.

### 2. BACKGROUND OF THE INVENTION

#### 20                   2.1. Mitochondrial Theory of Aging

                  The mitochondrial theory of aging proposes that accumulation of spontaneous somatic mutations of mitochondrial DNA (mtDNA) leads to errors of mtDNA-encoded polypeptide chains (Harman, 1956, J. Gerontol. 11:298-300; Harman, 1972, J. Am. Geriatr. Soc. 20:145-147; Miquel et al., 1980, Exp. Gerontol. 15:575-591; Linnane et al.,  
25                   1989, Lancet, I:642-645; Arnheim and Cortopassi, 1992, Mutat. Res. 275:157-167; Ozawa, 1995, Biochim. Biophys. Acta 1271:177-189; de Grey, 1997, BioEssays 19:161-166; de Grey, 1998, J. Anti-Aging Med. 1:53-66; Lenaz et al., 1997, Mol. Cell. Biochem. 174:329-333; and Lenaz et al., 1998, BioFactors 8:195-204). These errors occurring in mtDNA-encoded polypeptide chains are stochastic and randomly transmitted during mitochondrial  
30                   division and cell division. The consequence of these alterations, affecting exclusively the four mitochondrial complexes involved in protein translocation, is defective oxidative phosphorylation. Respiratory chain defects may become associated with increased oxidative stress, thus establishing a vicious cycle with amplification of the original damage (Ozawa, 1995, Biochim. Biophys. Acta 1271:177-189 and Lenaz, 1998, Biochim. Biophys.  
35                   Acta 1366:53-67). In this view, therefore, mutated mitochondrial DNA, despite being present only in very small quantities in the body, may be the main generator of oxidative stress. Mutations in mtDNA are mainly represented by deletions and are unevenly



distributed throughout the body. Each type of deletion is usually found at a very small percentage of total mtDNA. However, considering that the total number of deletions may be several hundreds in the different copies of mtDNA, they may account for such an amount to overcome the threshold required for decreasing the respiratory chain activity (Ozawa, 1995, *Biochim. Biophys. Acta* 1271:177-189; Yoneda et al., 1995, *Biochem. Biophys. Res. Comm.* 209:723-729; and Ozawa, 1997, *Physiol. Rev.* 77:425-464; Lenaz, 1998, *Biochim. Biophys. Acta* 1366:53-67 and Schon et al., 1996, *Cellular Aging and Cell Death*, J. Wiley & Sons, Inc., New York, pp. 19-34).

Aging was proposed to result from an ever-increasing level of destructive chemical reactions involving free radicals, with mitochondria as the principal mediators of the process (Harman, 1956, *J. Gerontol.* 11:298-300 and Harman, 1972, *J. Am. Geriatr. Soc.* 20:145-147). The main line of reasoning to support this idea is that, of all subcellular components, mitochondria is both a major source of free radicals and a major direct victim of free radical damage. As a result, loss of mitochondrial function may be the driving intracellular change underlying aging, and the cause of other pro-oxidant changes such as slower protein turnover. There is considerable indirect as well as direct experimental support for the theory. For example, a decline in ATP synthesis capacity and of energy-depending processes during aging has been reported (Syrový and Gutmann, 1997, *Exp. Gerontol.* 12:31-35; Sugiyama et al., 1993, *Biochem. Mol. Biol. Int.* 30:937-944; Boffoli et al., 1996, *Biochim. Biophys. Acta* 1226:73-82; and Lenaz et al., 1998, *BioFactors* 8:195-204).

The mitochondrial theory of aging is currently among the most popular theories of aging as it takes into account one of the most common sources of genetic lesions associated with senescence, that of mtDNA. Mitochondrial DNA is located at the inner mitochondrial membrane near the sites of formation of highly reactive oxygen species and their products. The mitochondrial genome encodes several subunits of the electron transport chain as well as components of the ATP synthase and mitochondrial tRNAs and rRNAs. Up to 2-4% of the oxygen metabolized by mitochondria is estimated to be converted to oxygen radicals because the flow process of electrons of the mitochondrial electron transport chain is not fully efficient (Boveris et al., 1972, *Biochem. J.* 128:617-630 and Richter et al., 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:6465-6467). In contrast to the nuclear genome, mtDNA may be unable to counteract the damage inflicted by those products of respiration because mitochondria lack excision and recombination repair (Miquel, 1992, *Mutat. Res.* 275:209-216). After an oxidative stress to cultured cells, the damage to mtDNA is higher and persists longer than that to nuclear DNA (Yakes and Van Houten, 1997, *Proc. Natl. Acad. Sci. U.S.A.* 94:514-519). The steady state level of mtDNA



oxidative change is about ten to sixteen times greater than that of nuclear DNA as indicated by the amount of 8-oxo-2'-deoxyguanosine (a biomarker of oxidative DNA damage) formed by the reaction of hydroxyl free radicals with guanine in mtDNA compared to nuclear DNA (Richter et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:6465-6467 and Shigenaga et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91:10771-10778). Also, lipid peroxidation of mitochondrial membranes seems to damage mtDNA as indicated by altered electrophoretic mobility (Balcavage, 1982, Mech. Aging Dev. 19:159-170).

However, alterations of mtDNA of themselves have been difficult to link to other forms of cellular and tissue changes related to aging. Chief among these is low density lipoprotein (LDL) oxidation and atherogenesis (Steinberg, 1997, J. Biol. Chem. 272:20963-20966).

## 2.2. Plasma Membrane Redox System

A consistent correlate of aging cells is the accumulation of somatic mutations of mitochondrial DNA (mtDNA) leading to defective oxidative phosphorylation through alterations that affect exclusively the four mitochondrial complexes involved in proton translocation (Harman, 1956, J. Gerontol. 11:298-300; Harman, 1972, J. Am. Geriatr. Soc. 20:145-147; Miquel et al., 1980, Exp. Gerontol. 15:575-591; Linnane et al., 1989, Lancet I:652-645; Arnheim and Cortopassi, 1992, Mutat. Res. 275:157-167; Ozawa, 1995, Biochim. Biophys. Acta 1271:177-189; de Grey, 1997, BioEssays 19:161-166; de Grey, 1998, J. Anti-Aging Med. 1:53-66; Lenaz et al., 1997, Mol. Cell. Biochem. 174:329-333; and Lenaz et al., 1998, BioFactors 8:195-204). A major piece of the puzzle missing from our information is how mitochondrial lesions are propagated to adjacent cells and blood components in the aging cascade. A plasma membrane oxido-reductase (PMOR) system has been suggested to augment survival of mitochondrially deficient cells through regeneration of oxidized pyridine nucleotide. The pyridine nucleotide is required to sustain glycolytic ATP production in the presence of diminished respiratory chain activity (de Grey, 1997, BioEssays 19:161-166; de Grey, 1998, J. Anti-Aging Med. 1:53-66); Yoneda et al., 1995, Biochem. Biophys. Res. Comm. 209:723-729; Schon et al., 1996, Cellular Aging and Cell Death, J. Wiley and Sons, New York, pp. 19-34; Ozawa, 1997, Physiol. Rev. 77:425-464; and Lenaz, 1998, BioFactors 8:195-204).

A cell surface protein with hydroquinone (NADH) oxidase activity (designated NOX) that functions as a terminal oxidase of PMOR has been discovered by the Inventors. Thus, a complete electron transport chain involving a cytosolic hydroquinone reductase, plasma-membrane located quinones and the NOX protein was elucidated by the Inventors (Kishi et al., 1999, Biochem. Biophys. Acta 1412:66-77 and Morré, 1998, Plasma

Membrane Redox Systems and their Role in Biological Stress and Disease, Klewer Academic Publishers, Dordrecht, The Netherlands, pp. 121-156). This system provides a rational basis for operation of the mitochondrial theory of aging and for propagation of aging related mitochondrial lesions, including a decline in mitochondrial ATP synthetic capacity and other energy-dependent processes during aging (Boffoli et al., 1996, Biochem. Biophys. Acta 1226:73-82; Lenaz et al., 1998, BioFactors 8:195-204; de Grey, 1997, BioEssays 19:161-166; and de Grey, 1998, J. Anti-Aging Med. 1:53-66).

Alterations in mitochondria DNA are by far the most common sources of genetic lesion associated with aging and senescence. It has been widely noted that mitochondrial DNAs are located at the inner mitochondrial membrane near sites where highly reactive oxygen species and their products might be formed. Several subunits of the electron transport chain as well as components of the ATP synthase and mitochondrial tRNAs and rRNAs are encoded by the mitochondrial genome. Since the flow of electrons of the mitochondrial electron transport chain is not fully efficient, up to 2-4% of the oxygen metabolized by mitochondria has been estimated to be converted to oxygen radicals (Boveris et al., 1972, Biochem. J. 128:617-630 and Richter et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:6465-6467). A major tenet of the mitochondrial theory of aging is that mtDNA may be unable to counteract the damage inflicted by oxygen radicals and their products due to a lack of excision and recombination repair mechanisms (Miquel, 1992, Mutat. Res. 275:209-216). This has been demonstrated in cultured cells where damage to mtDNA resulting from oxidative stress is not only higher but persists longer than does damage to nuclear DNA (Yakes and Van Houten, 1997, Proc. Natl. Acad. Sci. U.S.A. 94:514-519). Using the amount of 8-oxo-2'-deoxyguanosine as a bio-marker of oxidative DNA damage formed by the reaction of hydroxyl free radicals with guanine in mtDNA, the steady state level of mtDNA oxidative change is about 10 to 16 times greater than that of nuclear DNA (Richter et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:6465-6467 and Shigenaga et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91:10771-10778). Even lipid peroxidation of mitochondrial membranes seems to lead to damage of mtDNA (Balcavage, 1982, Mech. Aging Dev. 19:159-170).

Nevertheless, alterations of mtDNA and other forms of cellular and tissue changes, related to aging, have been difficult to link. Chief among these is the oxidation of low density lipoproteins (LDLs) and its implications as causal to atherogenesis (Steinberg, 1997, J. Biol. Chem. 272:20963-20966).

A model to link accumulation of lesions in mtDNA to extracellular responses, such as the oxidation of lipids in low density lipoprotein (LDLs) and the attendant arterial changes, was first proposed with rho<sup>0</sup> cells (Larm et al., 1994, J. Biol.



Chem. 269:30097-30100; Lawen et al., 1994, Mol. Aspects. Med. 15:s13-s27; de Grey, 1997, BioEssays 19:161-166; and de Grey, 1998, J. Anti-Aging Med. 1:53-66). These cells lack mtDNA and are unable to carry out oxidative phosphorylation. It has been demonstrated that the PMOR system actually functions to regenerate  $\text{NAD}^+$  from NADH (Larm et al., 1994, J. Biol. Chem. 269:30097-30100 and Lawen et al., 1994, Mol. Aspects. Med. 15:s13-s27). In the absence of functional mitochondrial respiratory chain, NADH accumulates as the result of glycolytic production of ATP. The  $\rho^0$  cells lacking functional mitochondria apparently survive though enhanced electron flow to molecular oxygen via PMOR. The PMOR is accordingly over-expressed in these cells. Oxidative stress and LDL oxidation are common complicating features in diabetics (Kennedy and Lyons, 1998, Metabolism 56:14-21).

The capacity of cells to generate ATP is determined either by reoxidation of NADH by mitochondrial respiratory mechanisms (reduction of pyruvate and uridine are provided, cells can grow without a functional mitochondrial electron transfer from oxygen to water) or by cytosolic glycolytic mechanisms (reduction of pyruvate to lactate). Transformed human cells in culture provided with excess pyruvate grow anaerobically on a glucose medium where  $\text{NAD}^+$  is regenerated from the NADH that is produced during glycolysis (Vaillant et al., 1996, J. Bioenerg. Biomemb. 28:531-540). This continual regeneration of  $\text{NAD}^+$  ensures that the glycolytic pathway will provide sufficient ATP to sustain cell growth and viability.

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### 2.3. Plasma Membrane Oxido-Reductase (PMOR) Chain: Role in Aging

Aging cells expressing mitochondrial lesions require a functional PMOR, as observed in  $\rho^0$  cells. Mitochondrial DNA encodes respiration and oxidative phosphorylation enzymes exclusively so that cells with functionally-deficient mitochondria become anaerobic if they are to survive. In such cells, the PMOR, as demonstrated by the Inventors, regenerate sufficient export of reducing equivalents to maintain the  $\text{NAD}^+/\text{NADH}$  homeostasis, ensuring survival of cells completely deficient in aerobic respiration.

Work by the Inventors done in collaboration with Prof. T. Kishi, Kobe-Gakuin University, Japan, has described a cell surface NADH oxidase protein, designated NOX, capable of oxidizing hydroquinones (Kishi et al., 1999, Biochem. Biophys. Acta 1412:66-77). This protein, which is located at the exterior of the cell, appears to be multifunctional but may have a major function as a terminal oxidase of the PMOR (Morré, 1995, Biochem. Biophys. Acta 1240:201-208 and DeHahn et al., 1997, Biochem. Biophys. Acta 1328:99-108). These findings define a complete electron transfer chain of the plasma membrane capable of transfer of electrons from NADH to an external electron acceptor via a reduced quinone intermediate.

The Inventors demonstrate that in cells where plasma membrane oxidoreductase (PMOR) is



over-expressed/activated electrons are transferred from NADH to external acceptors via a defined electron transport chain. The resultant transfer could result subsequently in the generation of reactive oxygen species (ROS) at the cell surface. Such cell surface-generated ROS then would be capable of propagating an aging cascade originating in mitochondria to both adjacent cells as well as to circulating blood components such as low density lipoproteins.

Mammalian plasma membranes are enriched in coenzyme Q (ubiquinone) and the plasma membrane at the cytosolic surface contains a quinone reductase capable of oxidizing NADH and reducing coenzyme Q. The electron acceptor is either molecular oxygen, or under certain conditions, both molecular oxygen and protein disulfides (Morré, 1994, *J. Bioenerg. Biomemb.* 26:421-433; Chueh et al., 1997, *J. Biol. Chem.* 272:11221-11227; and Morré et al., 1998, *J. Bioenerg. Biomemb.* 30:477-487). The enzyme can alternate between the two acceptors (Morré, 1998, *Plasma Membrane Redox Systems and their Role in Biological Stress and Disease*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 121-156). Hormones and growth factors stimulate NADH oxidation and favor protein disulfide reduction at the expense of oxygen consumption (Brightman et al., 1992, *Biochim. Biophys. Acta* 1105:109-117; Morré, 1994, *J. Bioenerg. Biomemb.* 26:421-433; and Chueh et al., 1997, *J. Biol. Chem.* 272:11221-11227). Stoichiometric relationships have been demonstrated among protein disulfide reduction, NADH oxidation and protein-thiol formation using isolated plasma membranes from a plant source stimulated by an auxin plant growth factor, 2,4-D (Chueh et al., 1997, *J. Biol. Chem.* 272:11221-11227). A similar stoichiometry has been shown for NADH oxidation in HeLa cells (Morré et al., 1998, *J. Bioenerg. Biomemb.* 30:477-487).

As a terminal oxidase of the PMOR electron transport chain, the NOX protein is responsible not only for maintaining  $\text{NAD}^+/\text{NADH}$  homeostasis in anaerobic cells but appears to play a role in the enhanced generation of ROS in aged cells expressing mitochondrial mutations. Oxygen appears to be the principal natural electron acceptor for cytosolic NADH oxidation in the resting state. However, a number of parameters, including metals (iron or copper), could interrupt the orderly two-electron flow to molecular oxygen that ordinarily forms water and initiate a one-electron process producing superoxide ( $\text{O}_2^-$ ) radicals (Table 3 in Section 6.2). Superoxide then initiates a reaction that generates  $\text{H}_2\text{O}_2$  and other aggressive oxidants such as the hydroxy radical ( $\text{OH}^\bullet$ ) (Pac and Skulachev, 1997, *Mol. Cell. Biochem.* 174:305-319). These ROS appear to be released into the environment to react with neighboring cells and circulating molecules such as LDL (Steinberg, 1997, *J. Biol. Chem.* 272:20963-20966).

Clearly, there is a need to find agents which reduce the ability of AR-NOX to generate reactive oxygen species (ROS) for the purposes of reducing or treating the resultant physiological conditions, such as oxidation of lipids in low density lipoprotein (LDLs) and attendant arterial changes.

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### 3. SUMMARY OF THE INVENTION

The present invention provides pharmaceutical compositions, methods of use, and pharmaceutical kits for the treatment of disorders resulting from oxidative changes in cells that result in aging by targeting an aging-related isoform of NADH oxidase (AR-NOX), shed  
10 into the sera by aging cells.

The invention is based in part, on the Inventors' discovery that ubiquinones inhibit the activity of an aging-related isoform of NADH oxidase (AR-NOX) shed into the sera by aging cells. The inhibition of AR-NOX by ubiquinones results in a decrease in the generation of reactive oxygen species by AR-NOX. A decrease in reactive oxygen species  
15 should result in a decrease of oxidative damage resulting from said reactive oxygen species.

In another embodiment, the invention comprises methods and compositions for screening assays to identify agents that sequester AR-NOX. In one embodiment, the invention encompasses methods for detecting cell-membrane associated AR-NOX and soluble AR-NOX in sera.

20 The pharmaceutical compositions further comprise varying modes of administration of compounds that sequester AR-NOX. The modes of administration of compounds includes but is not limited to capsules, tablets, soft gels, solutions, suppositories, injections, aerosols, or a kit. In yet another embodiment, the invention comprises the isolation and characterization of AR-NOX.

25

#### 3.1. Definitions

As used herein, the term "disorder" refers to an ailment, disease, illness, clinical condition, or pathological condition.

As used herein, the term "reactive oxygen species" refers to oxygen derivatives  
30 from oxygen metabolism or the transfer of free electrons, resulting in the formation of free radicals (e.g., superoxides or hydroxyl radicals).

As used herein, the term "antioxidant" refers to compounds that neutralize the activity of reactive oxygen species or inhibit the cellular damage done by said reactive species.

As used herein, the term "pharmaceutically acceptable carrier" refers to a  
35 carrier medium that does not interfere with the effectiveness of the biological activity of the active ingredient, is chemically inert, and is not toxic to the patient to whom it is administered.



As used herein, the term "pharmaceutically acceptable derivative" refers to any homolog, analog, or fragment corresponding to the ubiquinone formulations described in Section 5.1. *infra* which exhibits antioxidant activity and is relatively non-toxic to the subject.

The term "therapeutic agent" refers to any molecule, compound, or treatment, preferably an antioxidant, that assists in the prevention or treatment of the disorders, or complications of disorders caused by reactive oxygen species.

The term "agent that sequesters AR-NOX" refers to any molecule, compound, or treatment that interacts with AR-NOX, thus decreasing the reaction of AR-NOX with other substrates and inhibits the ability of AR-NOX to generate reactive oxygen species.

The antioxidants, cellular components, and target proteins defined herein are abbreviated as follows:

mitochondrial DNA	mtDNA
nicotinamide adenine dinucleotide	NADH
cell surface hydroquinone (NADH) oxidase with protein disulfide-thiol isomerase activity	NOX
NOX specific to non-cancer cells	CNOX
NOX specific to aged cells	AR-NOX
NOX specific to cancer cells	tNOX
low density lipoproteins	LDLs
plasma membrane oxido-reductase chain	PMOR
ubiquinone or coenzyme Q	CoQ
coenzyme Q <sub>10</sub>	Q <sub>10</sub>
reactive oxygen species	ROS

#### 4. BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Oxidation of reduced ubiquinol at 37° C by an enzymatic preparation with NADH oxidase activity solubilized from HeLa cells by low pH treatment, followed by heat and proteinase K. The disappearance of reduced ubiquinol was determined from the increase in absorbance at 410 nm as a function of time in the presence of 1 mg of the HeLa protein. The concentration of quinol was 50 mM, pH 7.

Fig. 2. The activity of the oxidase is periodic as shown for the oxidation of NADH by samples of sera from a young (A) and aged (B) patient. The maxima in the time course of NADH oxidation measured as a decrease in absorbance at 340 nm over 1 minute at 1.5 minute intervals marked by single arrows have an average period length of 24 minutes and are present in all sera thus far tested. In the aged subject, which is representative of both male and



female aged subjects 75 to 98, the maxima indicated by the double arrows reflect an average period length of about 26 min and are characteristic of a NOX isoform associated with aging.

Fig. 3. Time course of cytochrome c reduction by sera determined from the  $A_{550} - A_{540}$  determined at 10 sec intervals over 450 sec. After 200 sec either 15 mg superoxide dismutase (SOD) or 45 mg ubiquinone ( $Q_{10}$ ) were added and the reaction was continued. A. 40 year old female  $\pm$  SOD. B. 98 year old female  $\pm$  SOD. C. 83 year old female  $\pm$   $Q_{10}$ . D. 94 year old female  $\pm$   $Q_{10}$ . Results from multiple patients, both male and female, are summarized in Table 4 in Section 6.2.3. Line slopes are in nmoles/min/ml sera.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides pharmaceutical compositions, methods of use, and pharmaceutical kits for the treatment of disorders resulting from oxidative changes in cells that result in aging by targeting an aging-related isoform of NADH oxidase (AR-NOX), shed into the sera by aging cells.

The invention is based in part, on the Inventors' discovery that ubiquinones inhibit the activity of an aging-related isoform of NADH oxidase (AR-NOX) shed into the sera by aging cells. The inhibition of this hyperactive form of NOX, AR-NOX, by ubiquinones results in a decrease in the generation of reactive oxygen species by AR-NOX. A decrease in reactive oxygen species should result in a decrease of oxidative damage resulting from said reactive oxygen species.

### 5.1. Plasma Membrane Hydroquinone (NADH) Oxidase (NOX)

The plasma membrane NADH oxidase (NOX) is a unique cell surface protein with hydroquinone (NADH) oxidase and protein disulfide-thiol interchange activities that normally responds to hormone- and growth factors (Brightman et al., 1992, Biochim. Biophys. Acta 1105:109-117; Morré, 1994, J. Bioenerg. Biomemb. 26:421-433, and Morré, 1998, Plasma Membrane Redox Systems and their Role in Biological Stress and Disease, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 121-156). A hormone-insensitive and drug-responsive form of the activity designated tNOX also has been described which is specific for cancer cells (Bruno et al., 1992, Biochem. J. 284:625-628; Morré and Morré, 1995, Protoplasma 184:188-195; Morré et al., 1995, Proc. Natl. Acad. Sci. U.S.A. 92:1831-1835; Morré et al., 1995, Biochim. Biophys. Acta 1240:11-17; Morré et al., 1996, Eur. J. Cancer 32A:1995-2003; Morré et al., 1997, J. Biomemb. Bioenerg. 29:269-280; and U.S. Patent No. 5,605,810, which is incorporated by reference in its entirety for all purposes).

Because the NOX protein is located at the external plasma membrane surface and is not transmembrane, a functional role as an NADH oxidase is not considered likely (Morré, 1994, *J. Bioenerg. Biomemb.* 26:421-433; DeHahn et al., 1997, *Biochem. Biophys. Acta* 1328:99-108; and Morré, 1998, *Plasma Membrane Redox Systems and their Role in Biological Stress and Disease*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 121-156). While the oxidation of NADH provides a basis for a convenient method to assay the activity, the ultimate electron physiological donor appears to be hydroquinones with specific activities for hydroquinone oxidation greater than or equal to that of NADH oxidation and/or protein thiol-disulfide interchange (Kishi et al., 1999, *Biochem. Biophys. Acta* 1412:66-77).

The NOX protein partially purified from the surface of HeLa cells also exhibits ubiquinol oxidase activity (Kishi et al., 1999, *Biochem. Biophys. Acta* 1412:66-77). These preparations completely lack NADH: ubiquinone reductase activity and oxidize  $Q_{10}H_2$  at a rate of 3 to 6 nanomoles/min/mg protein. The  $K_m$  for reduced  $Q_{10}H_2$  is 30 mM. Activities are inhibited competitively by the cancer cell specific NADH oxidase inhibitors capsaicin (8-methyl-*N*-vanillyl-6-noneamide) and the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984) (Morré et al., 1995, *Proc. Natl. Acad. Sci. U.S.A.* 92:1831-1835; Morré et al., 1996, *Eur. J. Cancer* 32A:1995-2003; and Morré et al., 1995, *Biochim. Biophys. Acta* 1240:11-17). The oxidation of  $Q_{10}H_2$  proceeds with what appears to be a normal two-electron transfer in keeping with the participation of the plasma membrane NADH oxidase as a terminal oxidase of plasma membrane electron transport from cytosolic NAD(P)H via coenzyme Q to acceptors at the cell surface.

Evidence that NOX proteins under certain conditions are capable of the production of ROS is presented in Table 3 in Section 6.2.2. Ultraviolet light as a source of oxidative stress in cultured cells is used to initiate superoxide generation (Morré et al., 1999, *Biofactors* 9:179-187). Such generation is due to the NADH oxidase because in cell lines (HeLa, human cervical carcinoma and BT-20 human mammary carcinoma), that contain a capsaicin-responsive NADH oxidase, the response to UV is inhibited by capsaicin. In the MCF10A cell line (human mammary epithelia), a non-cancerous cell line lacking tNOX activity, the UV-induced generation of superoxide is unaffected by capsaicin and the resultant effects on the plasma membrane CNOX (Table 3 in Section 6.2.2). The switch whereby the oxidase may reduce oxygen by a one-electron or four-electron mechanism is not understood at present but may reside in a delicate redox balance of the carriers involved. Such a balance may be broken by oxidative stress or cell damage. Metal ions, such as iron and copper, released by tissue damage also may play a role (Hershko, 1992, *Molec. Aspects Med.* 13:113-165) in maintaining redox homeostasis.



## 5.2. Plasma Membrane Levels of Coenzyme Q

Plasma membrane ubiquinone or coenzyme Q (CoQ) plays a major role in the PMOR system. Ubiquinone or coenzyme Q (CoQ) occurs ubiquitously among tissues. In rat liver, the highest amount is found in the Golgi apparatus but it is also concentrated in the plasma membrane (Table 1) (Crane and Morré, 1977, Biomedical and Clinical Aspects of Coenzyme Q, Elsevier Scientific, Amsterdam-Oxford-New York, pp. 3-14 and Kalén et al., Lipids 25:93-99). The ubiquinone content of plasma membrane is two to five times that of microsomes and only half that of mitochondria.

Table 1. Distribution of ubiquinone in subcellular fractions from rat liver. The values are means  $\pm$  seven experiments (Kalén et al., 1987, Biochem. Biophys. Acta 926:70-78).

Fractions	Ubiquinone 9 (mg/mg protein)
Homogenate	$0.79 \pm 0.08$
Golgi apparatus	$2.62 \pm 0.15$
Lysosomes	$1.86 \pm 0.18$
Mitochondria	$1.40 \pm 0.16$
Inner mitochondrial membranes	$1.86 \pm 0.13$
Microsomes	$0.15 \pm 0.02$
Peroxisomes	$0.29 \pm 0.04$
Plasma membranes	$0.74 \pm 0.07$
Supernatant	$0.02 \pm 0.004$

Ubiquinone has long been considered to have both pro- and antioxidant roles over and above its more conventional role in mediating electron transport between NADH and succinic dehydrogenase and the cytochrome system of mitochondria (Ernster and Dallner, 1995, Biochim. Biophys. Acta 127:195-204 and Crane and Barr, 1985, Coenzyme Q, John Wiley & Sons, Chichester, pp. 1-37). Both pro- and antioxidant as well as electron transport roles should be considered for ubiquinone in the plasma membrane.

CoQ is normally a product of cellular biosynthesis and provides a potentially important source of one-electron pro-oxidant oxygen reduction (Andersson et al., 1994, Biochim. Biophys. Acta 1214:79-87 and Appelkvist et al., 1994, Molec. Aspects Med. 15S:37-46). In its reduced hydroquinone form (ubiquinol), it is a powerful antioxidant acting directly upon either superoxide or indirectly on lipid radicals alone or together with vitamin E ( $\alpha$ -tocopherol) (Crane and Barr, 1985, Coenzyme Q, John Wiley & Sons, Chichester, pp. 1-



37; Beyer and Ernster, 1990, Highlights of Ubiquinone Research, Taylor & Francis, London, pp. 191-213; and Beyer, 1994, J. Bioenerg. Biomemb. 26:349-358; Kagan et al., 1990, Biochem. Biophys. Res. Comm. 169:851-857; and Ernster et al., 1992, BioFactors 3:241-248).

5           The antioxidant action of ubiquinol normally yields the ubisemiquinone radical. The latter is converted back to ubiquinol by re-reduction through the electron transfer chain in mitochondria or by various quinone reductases in various cellular compartments including the plasma membrane (Takahashi et al., 1995, Biochem. J. 309:883-890; Takahashi et al., 1996, J. Biochem. (Tokyo) 119:256-263; Beyer et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:2528-2532; Beyer et al., 1997, Molec. Aspects Med. 18:s15-s23; Navarro et al., 1995, Biochem. Biophys. Res. Comm. 212:138-143; Villalba et al., 1995, Molec. Aspects Med. 18:s7-s13; and Arroyo et al., 1998, Protoplasma 205:107-113). Thus, ubiquinone may transform from a beneficial one-electron carrier to a superoxide generator if the ubisemiquinone anion becomes protonated (Nohl et al., 1996, Free Rad. Biol. Med. 20:207-213).

15           In perfused rat liver and in isolated rat hepatocytes, the anti-cancer quinone glycoside, adriamycin, induces oxidative stress by enhancing ROS production (Valls et al., 1994, Biochem. Mol. Biol. Int. 33:633-642 and Beyer et al., 1996, Proc. Natl. Acad. Sci. U.S.A. 93:2528-2532). Exogenous CoQ addition prevents this ROS production and concomitantly protects the cells from oxidative damage. Similar effects of exogenous CoQ on NOX-mediated ROS production have been observed (Table 3 in Section 6.2.2). The antioxidant effect at the plasma membrane may ameliorate LDL oxidation by scavenging ROS by PMOR produced at the cell surface (Thomas et al., 1997, Molec. Aspects Med. 18:s85-s103).

25           Some studies have shown that overall CoQ levels decrease with age (Beyer et al., 1985, Mech. Aging Dev. 32:267-281; Kalén et al., 1990, Lipids 25:93-99; and Genova et al., 1995, Biochem. J. 311:105-109). However this is not true for all tissues and especially for the brain, where high CoQ levels are maintained throughout aging (Söderberg et al., 1990, J. Neurochem. 54:415-423 and Battino et al., 1995, Mech. Aging Dev. 78:173-187). Thus, the invention also encompasses particular therapeutic levels of coenzyme Q for inhibiting or reducing the effects caused by overactive or aberrant cell surface PMOR system and for sequestering NOX isoforms.

### 5.3. Evidence for an Aging-Related CNOX Protein

35           The NOX protein is anchored in the outer leaflet of the plasma membrane (Morré, 1995, Biochem. Biophys. Acta 1240:201-208 and DeHahn et al., 1997, Biochem.

Biophys. Acta 1328:99-108). Subsequently, the activity was shown to be shed in soluble form from the cell surface (Morré et al., 1996, Biochim. Biophys. Acta 1280:197-206). The presence of the shed form in the circulation provides an opportunity to use patient sera as a source of the NOX protein for large scale isolation and characterization studies and to  
5 examine the NOX activity in sera of subjects of advanced age in a simple and non-invasive procedure that permits side-by-side comparisons with sera of young adults. A serum form of the CNOX activity which is specific to sera from elderly subjects (AR-NOX) has been identified by the Inventors. Results are shown in Table 4 in Section 6.2.3 for elderly individuals 80-94 years of age. This sera has a superoxide-generating and aging-related  
10 enzymatic activity, which is substantially reduced by addition of 0.1 mM coenzyme Q.

The source of the circulating age-related form of the superoxide-generating activity is hypothesized to result from shedding from cells, as observed in other NOX forms. Consistent with this interpretation was the appearance of a coenzyme Q inhibitable age-related reduction of ferric cytochrome c with a buffy coat fraction (lymphocytes) comparing young  
15 and aged patients.

Thus, in one embodiment, the invention is directed to utilizing drugs which sequester, neutralize, bind, or otherwise block or eliminate, the AR-NOX protein and inhibit its ability to generate reactive oxygen species so that the cells undergo apoptosis (Morré et al., 1995, Proc. Natl. Acad. Sci. U.S.A. 92:1831-1835; Vaillant et al., 1996, J. Bioenerg.  
20 Biomemb. 28:531-540; and Dai et al., 1997, Mol. Cell. Biochem. 166:101-109). Additionally, based on the presence of an age related PMOR system capable of generating ROS at the cell surface, an approach to ablation of anaerobic cells in aged tissues is feasible. The benefits of such an approach include the fact that: (1) while normally only a small percentage of muscle fibers become anaerobic even in severely affected tissues, the  
25 elimination of these cells would not be expected to have deleterious side effects; (2) apoptosis of anaerobic cells results in the of lowering serum levels of oxidized lipoproteins and an overall reduction of the oxidative stress to surrounding healthy cells. The cells displaying all of the characteristics listed *supra* are hereby defined as aged cells. Generally, the characteristics of aged cells includes those that express and/or shed AR-NOX, and include, but  
30 are not limited to, those exhibiting one or more of the following characteristics: an age-related PMOR system, the ability to generate reactive oxygen species, and have functionally defective mitochondria.

In another embodiment, the invention is directed to utilizing agents, *e.g.*, drugs or supplements, which switch the NOX protein from oxygen reduction to protein disulfide  
35 reduction. The advantage of such an approach has already been observed with plant cells in response to auxins (Chueh et al., 1997, J. Biol. Chem. 272:11221-11227).



#### 5.4. Methods of Detecting AR-NOX

The invention further contemplates using AR-NOX as a diagnostic tool when oxidative damage to cells and/or tissue is suspected. As such, AR-NOX in tissue, cells, or circulation may be detected. In one embodiment, detection may be achieved

5 immunologically, by employing antibodies specific to AR-NOX (described *supra* in Section 5.4). Said antibodies may be conjugated to a wide variety of labels, *e.g.*, radioisotopes, enzymes, fluorescers, chemilumescers, and the like, wherein the label provides a detectable signal.

Alternatively, in another embodiment, detection is based upon assays that  
10 recognize that sera with AR-NOX exhibits a higher rate of cytochrome c reduction than sera without AR-NOX. In this embodiment, AR-NOX reacts with a substrate capable of generating reactive oxygen species, *e.g.*, *superoxide dismutase*, which results in cytochrome c reduction. The detection of cytochrome c may be detected spectrophotometrically by measuring the absorbance at about 540 nm to 550 nm.

15 In yet another embodiment, AR-NOX is detected in an assay which measures the disappearance of the ascorbate radical spectrophotometrically by measuring the absorbance at about 265 nm since AR-NOX reduces an electron acceptor, *e.g.*, ascorbate radical. In another embodiment, a similar spectrophotometric assay may be carried out by measuring the reduction of  $\text{NAD}^+$  by AR-NOX using methods known in the art (Morre et al.,  
20 1995, Proc. Natl. Acad. Sci. U.S.A. 92:1831-1835).

Other embodiments of the invention include assays based on the unique  
oscillation property of AR-NOX since NOX from healthy cells and AR-NOX exhibit varying oscillations, which are given by the oxidation of NADH, ubiquinol, or reduced vitamin  $\text{K}_1$ . As detailed in Section 6.2.3., varying oscillations have been observed for several NOX  
25 activity forms such that the oscillations serve as a diagnostic feature to identify NOX activity forms during their purification (Morre, 1998, Plasma Membrane Redox Systems and their Role in Biological Stress and Disease, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 121-156, which is incorporated by reference in its entirety).

In yet another embodiment of the invention, AR-NOX is detected by resistance  
30 to retinoic acid, since NOX from healthy cells is inhibited by retinoic acid and AR-NOX is not inhibited by retinoic acid (Morre, 1998, Plasma Membrane Redox Systems and their Role in Biological Stress and Disease, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 121-156).

Still other embodiments include a method using AR-NOX to identify cells  
35 where mitochondrial functions are depressed and consequently, PMOR is overexpressed. Such cells may be identified in the presence of overexpressed AR-NOX. In cells where



PMOR is overexpressed as a result of decreased electron input from the respiratory chain, overcompensation by AR-NOX may represent a diagnostic feature.

AR-NOX may also be assayed for disulfide-thiol interchange activity, by using dithio-dipyridyl substrates. Examples of derivatives of dithio-dipyridyl substrates include 2-pyrimidine-2-thione, 2, 2'-dithiopyridine, and 6,6'-dithionicotinic acid, which may be used to assay the disulfide-thiol interchange activity of AR-NOX (Morré et al., 1999, Mol. Cell. Biochem. 200:7-13).

### 5.5. Methods of Identifying Agents that Interact with AR-NOX

The present invention relates to *in vitro* and *in vivo* methods for screening for agents which target AR-NOX. Within the broad category of *in vitro* selection methods, several types of methods are likely to be particularly convenient and/or useful for screening test agents. These include, but are not limited to, methods which measure a binding interaction between two or more components, and methods which measure the activity of an enzyme which is one of the interacting components, *i.e.*, AR-NOX.

Binding interactions between two or more components can be measured in a variety of ways known in the art. One approach is to label one of the components with an easily detectable label, place it together with the other component(s) in conditions under which they would normally interact (*e.g.*, ubiquinone), perform a separation step which separates bound labeled component from unbound labeled component, and then measure the amount of bound component. The effect of a test agent included in the binding reaction can be determined by comparing the amount of labeled component which binds in the presence of this agent to the amount which binds in its absence.

The test agent may be labeled with a detectable marker, using methods for labeling known in the art. A "detectable marker" refers to a moiety, such as a radioactive isotope or group containing same, or nonisotopic labels, such as enzymes, biotin, avidin, streptavidin, digoxigenin, luminescent agents, dyes, haptens, and the like. Luminescent agents, depending upon the source of exciting energy, can be classified as radioluminescent, chemiluminescent, bioluminescent, and photoluminescent (including fluorescent and phosphorescent).

The separation step in this type of approach can be accomplished in various ways. In one approach, (one of) the binding partner(s) for the labeled component can be immobilized on a solid phase prior to the binding reaction, and unbound labeled component can be removed after the binding reaction by washing the solid phase. Attachment of the binding partner to the solid phase can be accomplished in various ways known to those skilled in the art, including but not limited to, chemical cross-linking, non-specific adhesion to a

plastic surface, interaction with an antibody attached to the solid phase, interaction between a ligand attached to the binding partner (e.g., biotin), and a ligand-binding protein (e.g., avidin or streptavidin) attached to the solid phase.

Alternatively, the separation step can be accomplished after the labeled  
5 component has been allowed to interact with its binding partner(s) in solution. If the size differences between the labeled component and its binding partner(s) permit such a separation, the separation can be achieved by passing the products of the binding reaction through an ultrafilter whose pores allow passage of unbound labeled component but not of its binding partner(s) or of labeled component bound to its partner(s). Separation can also be achieved  
10 using any reagent capable of capturing a binding partner of the labeled component from solution, such as an antibody against the binding partner, a ligand-binding protein which can interact with a ligand previously attached to the binding partner.

Another *in vitro* selection method which may be used is the screening of combinatorial chemistry libraries, using ubiquinone or ubiquinone derivatives as a base  
15 molecule. The methods for the generation and screening of combinatorial libraries are described in U.S. Patent No. 5,565,324, which is incorporated by reference in its entirety. Briefly, the synthesis of the ubiquinone derivatives, using combinatorial chemistry, involves syntheses with a plurality of stages, wherein each stage involves a plurality of choices, where large numbers of products with varying compositions are obtained. The substrates carrying  
20 the final product compounds may be screen for AR-NOX binders.

Test methods which rely on measurements of AR-NOX enzymatic activity have been described in the previous subsection, *supra*.

The invention also comprises *in vivo* screening methods to identify test agents that interact with AR-NOX. In this approach, coding sequences encoding part or all of a  
25 component(s) would be introduced into a selected type of cell. Coding sequences for this approach include cloned genes, cDNAs, fragments of either, fragments amplified by the polymerase chain reaction, natural RNAs, transcribed RNAs, or the like. For example, coding sequences for two or more components which are known to interact with each other (e.g., AR-NOX and ubiquinone) are introduced into a cell, and agents are tested for their ability to  
30 moderate and/or displace the interaction between these two components.

In another embodiment, proteins that interact with AR-NOX may be identified by a yeast two-hybrid assay (Fields and Song, 1989, Nature 340:245-246). The yeast two-hybrid assay takes advantage of the properties of the GAL4 protein of the yeast *Saccharomyces cerevisiae*. GAL4 is a transcriptional activator required for the expression of  
35 genes encoding enzymes of galactose utilization, which consists of two separable and functionally essential domains: an N-terminal domain which binds to specific DNA sequences



(UASG) and a C-terminal domain containing acidic regions, which is necessary to activate transcription. The two-hybrid screen comprises a system of two hybrid proteins containing parts of GAL4: the GAL4 DNA-binding domain fused to a protein 'X' and a GAL4 activating region fused to a protein 'Y'. If X and Y can form a protein-protein complex and reconstitute proximity of the GAL4 domains, transcription of a gene regulated by UASG (e.g.,  $\beta$ -galactosidase) occurs. In this embodiment, AR-NOX may be used as the "bait" to screen for the "prey," i.e., putative interacting agents, present as cDNAs from a cDNA library. In both cases, the AR-NOX and putative interacting agents are fused in frame with the two parts of GAL4, or other transcriptional activator. Kits for two-hybrid assays are readily available, e.g., Hybrid Hunter™ Two-Hybrid system from Invitrogen.

Gene therapy approaches may also be used in accordance with the present invention to inhibit AR-NOX. Among the compounds which may interact with AR-NOX, and therefore disrupt its activity, are antisense and ribozyme molecules. Such molecules are designed to inhibit the expression of the target gene, AR-NOX. Techniques for the production and use of antisense and/or ribozyme molecules are well known to those of skill in the art and can be designed with respect to the nucleotide sequence of AR-NOX.

Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxynucleotides derived from the translation initiation site, e.g., between the -10 and +10 regions of the target gene nucleotide sequence of interest, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of mRNA (reviewed in Rossi, 1994, Current Biology 4:469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme to complementary target RNA, followed by an endonucleolytic cleavage. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the catalytic sequence responsible for mRNA cleavage (U.S. Patent No. 5,093,246).

The invention further encompasses methods for monitoring patient response to the agents identified by the methods described *supra*. By monitoring circulating AR-NOX activity in patient sera, it will be possible to determine therapeutic dosages and to monitor therapeutic benefit. The response to the subject compositions may be monitored by assaying the blood or urine of the patient for the AR-NOX activity that is responsive to the agents that interact with AR-NOX. By following the above monitoring procedures, an effective dosage of the subject compositions may be administered in accordance with the requirement of the individual patient.

### 5.6. Inhibition of AR-NOX by Ubiquinones

The invention comprises the administration of a therapeutically effective amount of ubiquinones to a patient with a disorder or a complication of a disorder caused by oxidative damage resulting from the generation of reactive oxygen species by AR-NOX. In a preferred embodiment, the total daily amount administered is from about 1 to about 500 mg of ubiquinones. In a more preferred embodiment, the total daily amount administered is from about 1 to 100 mg of ubiquinones.

The invention is based on studies by the Inventors that have identified a serum form of AR-NOX which is specific to sera from elderly patients, and absent from sera of younger patients. Not only is there a superoxide-generating and aging-related enzymatic activity present in sera of the elderly patients, but also the aging-related enzymatic activity is reduced by the addition of ubiquinone.

In one embodiment, the invention is used to identify patients suffering from disorders associated with reactive oxygen species who may be responsive to treatment with ubiquinones. Such responsive patients may be identified by assay of serum or urine for ubiquinone responsive superoxide generation. The generation of superoxide may be followed by reduction of cytochrome c (described in Section 6.1.3) or any other suitable biological or chemical method.

The ubiquinones are benzoquinones with a base structure of 2,3-dimethoxy-5-methylbenzoquinone nucleus ("Q<sub>n</sub>") and differ in the number of carbon atoms in the side chain of the 6-position, wherein n is the number of carbon units in the side chain (n is 1 to 12). The differences in properties are due to the difference in length of the side chain (Merck Index 10<sup>th</sup> Edition, 1983, Merck & Co., Inc., Rahway, NJ, p. 1407). Naturally occurring derivatives of ubiquinone are the coenzymes Q<sub>6</sub> to Q<sub>10</sub>, wherein Q<sub>10</sub> is naturally occurring in humans and Q<sub>9</sub> is naturally occurring in rats.

The invention comprises treating a patient with a pharmacologically effective amount of ubiquinones to inhibit the generation of reactive oxygen species. In a preferred embodiment, the ubiquinones are of the human derivative Q<sub>10</sub>. In another embodiment, the ubiquinones comprise the naturally occurring derivatives Q<sub>6</sub>, Q<sub>7</sub>, Q<sub>8</sub>, and Q<sub>9</sub>. In another embodiment, the ubiquinones comprise other derivatives Q<sub>1</sub>, Q<sub>2</sub>, Q<sub>3</sub>, Q<sub>4</sub>, Q<sub>5</sub>, Q<sub>11</sub>, and Q<sub>12</sub>. In another embodiment, the invention comprises mixtures of the ubiquinone derivatives described *supra*. The invention further comprises all pharmaceutically acceptable derivatives of the compositions listed *supra* for methods of treating a patient with an AR-NOX related disorder, with ubiquinone administration in the range of 0.1 to 100 mg per kg body weight.

In addition, the invention provides a method for screening for test compounds that interact with AR-NOX further comprising comparing the interaction of AR-NOX of the



test compound to the interaction of AR-NOX with ubiquinone, wherein the interaction of AR-NOX with ubiquinone serves as a positive control.

The invention also encompasses methods for monitoring patient response to ubiquinones. By monitoring circulating AR-NOX activity in patient sera, it will be possible to  
5 determine therapeutic dosages and to monitor therapeutic benefit from ubiquinones. The response to the subject compositions may be monitored by assaying the blood or urine of the patient for the AR-NOX activity that is responsive to the ubiquinone compositions. By following the above monitoring procedures, an effective dosage of the subject compositions may be administered in accordance with the requirement of the individual patient.

10

### 5.7. Isolation and Characterization of AR-NOX

The AR-NOX protein has been purified by the Inventors from lymphocytes and sera from aged individuals. The AR-NOX protein was purified by standard protein preparative techniques well known to those skilled in the art including ammonium sulfate  
15 precipitation, anion exchange, gel filtration, preparative SDS-PAGE and hydrophobic interaction chromatography, HPLC, and FPLC (Morré et al., 1996, Biochem. Biophys. Acta 1280)197-206 and Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Intersciences, N.Y.). The ability to generate superoxide as evidenced by reduction of cytochrome c and inhibition by coenzyme Q were used as selection criteria. Sufficient AR-  
20 NOX amino acid sequence was obtained by the Inventors to generate degenerate oligonucleotide primers for the amplification of a portion of the AR-NOX nucleic acid sequence.

The Inventors have also generated a NOX-specific polyclonal antibody to the AR-NOX protein from lymphocytes. Once the amino acid sequence of AR-NOX is deduced  
25 from the corresponding cDNA sequence, the amino acid sequence may be used to strategically generate peptide sera with therapeutic potential as probes specific to AR-NOX to investigate and ameliorate NOX responses to aging. Three criteria of the known protein sequence may be used in selecting sequences for antibody production: 1. hydrophilicity as calculated according to the algorithm of Hopp and Woods (Hopp and Woods 1981, Proc. Natl. Acad.  
30 Sci. U.S.A. 78:3824-3828); 2. surface probability as calculated according to the formula of Emini et al. (Emini et al., 1985, J. Virol. 53:836-839); and 3. the antigenic index measuring the probability that a region is antigenic as calculated by summing several weighted measures of secondary structure (Jamieson and Wolf, 1988, CABIOS 4:181-186). Peptide antibodies can be affinity-purified using immobilized peptide. The peptide antisera may be employed in  
35 co-incubation experiments with isolated lymphocytes from aged individuals and isolated

lipoprotein particles to demonstrate that specific AR-NOX inhibition can ablate propagation of oxidative stress in both *in vitro* and *in vivo* systems.

The present invention also relates to methods for cloning of AR-NOX. Using methods which are well known to those skilled in the art, recombinant cDNA libraries may be constructed using RNA prepared from cells known to express AR-NOX. The cDNA libraries may be constructed using a variety of vector systems, including but not limited to, bacteriophage vectors, plasmid vectors or mammalian expression vectors. See, for example, the techniques described in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. and *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Intersciences, N.Y. Alternatively, a human cDNA library library may be obtained from a commercial source, *e.g.*, Stratagene.

The recombinant cDNA libraries may be screened using a number of different techniques which are well known to those skilled in the art. For example, a mixture of degenerate oligonucleotide probes may be designed utilizing the partial amino acid sequence of AR-NOX. The oligonucleotides may be labeled and used directly to screen a cDNA library for clones containing inserts with sequence homology to the oligonucleotide sequences. Alternatively, the oligonucleotides may be used as primers in a polymerase chain reaction. The template for the reaction is cDNA may be obtained by reverse transcription of mRNA prepared from cells known to express AR-NOX, *i.e.*, lymphocytes from aged individuals. Alternatively, the template may be cDNA from a human cDNA library library obtained from a commercial source, *e.g.*, Stratagene. The amplified DNA fragment may be labeled and used to screen a library for isolation of full length clones. In another example, an expression library may be immunologically screened using antibodies directed against AR-NOX. In yet another embodiment of the invention, a cDNA library may be engineered into a mammalian expression vector and screened by transfection into the appropriate mammalian cell line followed by assaying for AR-NOX activity in the tissue culture supernatant.

In yet another embodiment of the invention, a method for cloning AR-NOX by means of polymerase chain reaction may be used to clone a cDNA coding for AR-NOX. Such a method may be utilized using RNA prepared from lymphocytes of aged individuals. Alternatively, AR-NOX may be cloned by polymerase chain reaction amplification of a human cDNA library obtained from a commercial source (*e.g.*, Stratagene).

In addition, gene expression assays using gene expression arrays or microarrays are now practicable for identifying changes in gene expression patterns between different cells or tissue types (see, *e.g.*, Schena et al., 1995, *Science* 270:467-470; Lockhart et al., 1996, *Nature Biotechnology* 14:1674-1680; and Blanchard et al., 1996, *Nature Biotechnology* 14:1649). Thus, in another, alternative embodiment of the invention, such



gene expression arrays or microarrays may be used to compare mRNA expression patterns in cells that exhibit AR-NOX activity (*e.g.*, as determined by one of the assays of the present invention) to mRNA expression patterns in cells that do not exhibit AR-NOX activity and thus, do not express AR-NOX.

5

### 5.8. Target Disorders

Disorders that can be treated by the methods of the present invention include any clinical condition in which oxidative species have been implicated.

Examples of clinical conditions in which oxidative species have been implicated include, but are not limited to, ischemia-reperfusion injury (*e.g.*, stroke/myocardial infarction and organ transplantation), cancer, aging, arthritis associated with age, fatigue associated with age, alcoholism, red blood cell defects (*e.g.*, favism, malaria, sickle cell anemia, Fanconi's anemia, and protoporphyrin photo-oxidation), iron overload (*e.g.*, nutritional deficiencies, Kwashiorkor, thalassemia, dietary iron overload, idiopathic hemochromatosis), kidney (*e.g.*, metal ion-mediated nephrotoxicity, aminoglycoside nephrotoxicity, and autoimmune nephrotic syndromes), gastrointestinal tract (*e.g.*, oral iron poisoning, endotoxin liver injury, free fatty acid-induced pancreatitis, nonsteroidal antiinflammatory drug induced gastrointestinal tract lesions, and diabetogenic actions of alloxan), inflammatory-immune injury (*e.g.*, rheumatoid arthritis, glomerulonephritis, autoimmune diseases, vasculitis, and hepatitis B virus), brain (*e.g.*, Parkinson's disease, neurotoxins, allergic encephalomyelitis, potentiation of traumatic injury, hypertensive cerebrovascular injury, and vitamin E deficiency), heart and cardiovascular system (*e.g.*, atherosclerosis, adriamycin cardiotoxicity, Keshan disease (selenium deficiency) and alcohol cardiomyopathy, eye (*e.g.*, photic retinopathy, ocular hemorrhage, cataractogenesis, and degenerative retinal damage), amyotrophic lateral sclerosis, and age-related macular degeneration (Slater, 1989, Free Rad. Res. Comm. 7:119-390; Deng et al., 1993, Science 261:1047-1051; Seddon et al., 1994, JAMA 272:1413-1420; Brown, 1995, Cell 80:687-692; and Jenner, 1991, Acta Neurol. Scand. 84:6-15).

The invention is also directed to preventing or alleviating complications of diabetes, atherogenesis, atherosclerosis, and related diseases. Oxidative stress and LDL oxidation are common complicating features in diabetics and circulating AR-NOX offers opportunities for redox modulation of blood constituents important to aging, atherogenesis, and atherosclerosis (Kennedy and Lyons, 1998, Metabolism 56:14-21).

In one embodiment, the invention is directed towards a method of preventing a complication of a primary disorder in patients wherein said complication results from oxidative damage resulting from the generation of reactive oxygen species by AR-NOX. The

method comprises administering to a patient with a primary disorder, in an amount effective to prevent said complication, an agent or agents that sequesters AR-NOX, in a pharmaceutically acceptable carrier.

5 In another embodiment, the invention is directed towards a method of preventing a secondary disorder in patients having a primary disorder that causes oxidative damage resulting from the generation of reactive oxygen species by AR-NOX. The method comprises administering to a patient having a primary disorder, in an amount effective to prevent said secondary disorder, an agent or agents that sequesters AR-NOX, in a pharmaceutically acceptable carrier.

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### 5.9. Pharmaceutical Formulations

Agents that interact with AR-NOX identified by the methods listed *supra* may be formulated into pharmaceutical preparations for administration to mammals for prevention or treatment of disorders in which oxidative species have been implicated. In a preferred  
15 embodiment, the mammal is a human.

Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may be prepared, packaged, and labeled for treatment.

If the complex is water-soluble, then it may be formulated in an appropriate buffer, for example, phosphate buffered saline or other physiologically compatible solutions.  
20 Alternatively, if the resulting complex has poor solubility in aqueous solvents, then it may be formulated with a non-ionic surfactant such as Tween, or polyethylene glycol. Thus, the compounds and their physiologically acceptable solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral, rectal administration or, in the case of tumors, directly injected into a solid tumor.

25 For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats);  
30 emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinyl  
35 pyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica);



disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well-known in the art.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

- 5 For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

- For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*,  
10 dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

- 15 The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or  
20 dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

- 25 The compounds may also be formulated as a topical application, such as a cream or lotion.

- In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection.  
30 Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

- The compositions may, if desired, be presented in a pack or dispenser device  
35 which may contain one or more unit dosage forms containing the active ingredient. The pack

may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The invention also provides kits for carrying out the therapeutic regimens of the invention. Such kits comprise in one or more containers therapeutically or prophylactically effective amounts of the compositions in pharmaceutically acceptable form. The composition in a vial of a kit of the invention may be in the form of a pharmaceutically acceptable solution, *e.g.*, in combination with sterile saline, dextrose solution, or buffered solution, or other pharmaceutically acceptable sterile fluid. Alternatively, the complex may be lyophilized or desiccated; in this instance, the kit optionally further comprises in a container a pharmaceutically acceptable solution (*e.g.*, saline, dextrose solution, etc.), preferably sterile, to reconstitute the complex to form a solution for injection purposes.

In another embodiment, a kit of the invention further comprises a needle or syringe, preferably packaged in sterile form, for injecting the complex, and/or a packaged alcohol pad. Instructions are optionally included for administration of compositions by a clinician or by the patient.

## 6. EXAMPLE: A MULTIFUNCTIONAL UBIQUINOL OXIDASE OF THE EXTERNAL CELL SURFACE AND SERA

### 6.1. Materials and Methods

#### 6.1.1. Measurement of Oxidation of Ubiquinol and of NADH Oxidation

Ubiquinol oxidation was estimated based on increase in absorbance at 410 nm for ubiquinone (Fig. 1). NADH oxidation was determined from the decrease in  $A_{340}$  measured spectrophotometrically as described (Brightman et al., 1992, Biochim. Biophys. Acta 1105:109-117).

#### 6.1.2. Superoxide generation by Cells and Response to UV

The generation of superoxide radical was determined by assaying the rate of superoxide dismutase (SOD)-inhibitable cytochrome c reduction (Mayo and Curnette, 1990, Meth. Enzymol. 186:567-575 and Butler et al., 1982, J. Biol. Chem. 257:10747-10750). The cytochrome c was from horse heart mitochondria (type VI, Sigma) and was dissolved in PBSG buffer (see below) to make a solution with a concentration of 1 mg/ml. Air-saturated reaction mixtures of 100 ml cytochrome c stock solution and 50 ml of a cell suspension (suspended in PBSG buffer) to give a final concentration of  $\sim 5 \times 10^6$  cells/ml were added to 2 ml of PBSG buffer (138 mM NaCl, 2.7 mM KCl, 8.1 mM  $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ , pH 7.4 supplemented with 0.9 mM  $\text{CaCl}_2$ , and 7.5 mM glucose and contained in plastic cuvettes. The formation of reduced



cytochrome c was measured in the presence and absence of SOD (15 mg, Sigma) or capsaicin (2.5 mM) by comparing the absorbance of the mixture at 550 nm – 540 nm. Superoxide formation was stimulated by using a handheld UV light (254 nm, 200 mw/cm<sup>2</sup>). The extent of cytochrome c reduction was monitored spectrophotometrically at 550 nm every ten sec with gentle mixing between readings. Data were analyzed from the slope of the change in a 550 nm – 540 nm before and after UV and then again after SOD or capsaicin was added. Data were expressed as nmoles O<sub>2</sub>/10<sup>6</sup> cells using a value of  $S_{M550nm} = 19.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ .

### 6.1.3. Cytochrome c Reduction by Sera and Inhibition by Ubiquinone (Q<sub>10</sub>)

Cytochrome c reduction by sera was assayed as for cells except that the samples were not mixed in between readings. The Q<sub>10</sub> was added as an ethanolic solution.

Sera were obtained from the patient population of St. Elizabeth Hospital, Lafayette, Indiana and the resident population of St. Anthony's Health Care, Lafayette, Indiana. Confidentiality of medical records was assured by assigning a number to each sample.

## 6.2. Results

### 6.2.1. Characteristics of NOX

Characteristics of the surface quinol oxidase (Fig. 1) are summarized in Table 2. The protein is of relatively low abundance and specific activity but sufficient to catalyze a substantial flow of electrons from cytosolic reduced pyridine nucleotide to molecular oxygen. Molecular oxygen has been shown to represent a physiological electron acceptor for the oxidase. However, under certain conditions, protein disulfides also may function as acceptors (Morre, 1994, J. of Bioenerg. and Biomemb. 26:421-433).

Table 2. Properties of NOX-catalyzed oxidation of ubiquinol.

	pH optimum	7.0
	EC <sub>50</sub> LY181984 <sup>a</sup> inhibition	30 nM (competitive)
5	EC <sub>50</sub> capsaicin <sup>b</sup> inhibition	1 nM (competitive)
	Cyanide	Resistant
	NOX-specific monoclonal antibodies	Inhibited
	K <sub>m</sub> for quinol	33 mM
10	V <sub>max</sub> (nmoles/min/mg pro)	3
	Oxygen consumption (nmoles/min/mg/pro)	2.5

<sup>a</sup>N-(4-methylphenylsulfonyl)-N'-(4-chlorophenyl)urea<sup>b</sup>8-methyl-N-vanillyl-6-noneamide

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**6.2.2. *In Vitro* Generation of Reactive Oxygen Species**

Cultured cells were subjected to ultraviolet light to perturbate electron flow to show that if the orderly two electron flow to molecular oxygen or protein thiols was suitably interrupted, a one electron process producing superoxide results. Such production leads to formation of hydrogen peroxide and possibly other oxidants such as hydroxide radical. These reactive oxygen species could then be released into the environment to react with neighboring cells and circulating molecules such as low density lipoproteins (LDLs). Results indicate that based on superoxide SOD-inhibitable reduction of cytochrome c, superoxide was generated by all three cell lines tested (Table 3). The SOD-inhibitable reduction of cytochrome c is assumed to be due at least partially to the cell surface NADH oxidase based on drug responsiveness. The HeLa (human cervical carcinoma) and BT-20 (human mammary adenocarcinoma) cells contain an activity form of the ubiquinol oxidase that is drug responsive (Morré et al., 1995, Proc. Natl. Acad. Sci. U.S.A. 92:1831-1835). Among the inhibitors that are specific to the cancer activity form are putative quinone site inhibitors such as capsaicin and the antitumor sulfonylurea LY181984 (Morré et al., 1995, Proc. Natl. Acad. Sci. U.S.A. 92:1831-1835). The MCF-10A (noncancerous human mammary epithelia) cells that lack the capsaicin-responsive NADH oxidase also lack the capsaicin-responsive UV-induced generation of superoxide (Morré et al., 1995, Proc. Natl. Acad. Sci. U.S.A. 92:1831-1835).

35



Table 3. Superoxide production (reduction of cytochrome c) by cell lines in response to UV irradiation and inhibition by superoxide dismutase (SOD) and by capsaicin.

Reduction of cytochrome c as a measure of superoxide formation, nmoles/min/10 <sup>6</sup> cells				
Cell line <sup>1</sup>	Initial	After UV <sup>2</sup>		
		No Addition	+ SOD <sup>3</sup>	+ Capsaicin <sup>4</sup>
HeLa S	0.8 ± 0.16	4.0 ± 1.0	1.1	0.8
BT-20	0.7 ± 0.2	5.1 ± 2.1	- 0.1	- 3.7
MCF 10A	1.5 ± 0.2	7.2 ± 0.1	- 0.7	7.2

<sup>1</sup>HeLa S, human cervical carcinoma; BT-20, human mammary adenocarcinoma; MCF 10A, human mammary epithelia (non-cancer).

<sup>2</sup>10 minutes of 254 nm, 200 mw/cm<sup>2</sup>

<sup>3</sup>15 mg (Sigma)

<sup>4</sup>2.5 mM added in DMSO. Rates were corrected for a DMSO blank (0.1% final concentration)

### 6.2.3. Evidence for AR-NOX

A partially-purified preparation of the oxidase from HeLa cells also responds to UV by generation of SOD- and capsaicin-inhibited superoxide, suggesting that the effect of UV is directly on the oxidase. It has previously been suggested that NADPH oxidase may be a source of reactive oxygen species generated by UV irradiation based on inhibition by diphenyliodonium (Gorman et al., 1997, FEBS Lett. 404:27-33).

The Inventors have identified a serum form of AR-NOX that is specific to sera from elderly subjects and low or absent from sera of younger subjects (Fig. 2). Sera contains NADH oxidase activities with properties similar if not identical to those of the NADH oxidase found at the cell surface (Morre et al., 1997, Archives Biochem. Biophys. 342:224-230). The invention contemplates the characterization and cloning of AR-NOX from the sera of aging patients. The invention further contemplates large scale isolation and purification of AR-NOX activity in the sera of subjects of advanced age in a simple and non-invasive procedure for side-by-side comparisons with sera of young adults. Such isolation and purification methods are known in the art (Chueh et al., 1997, Archives Biochem. Biophys. 342:38-47).

Based on results of Table 3, the ability of sera to reduce cytochrome c was measured. Sera of aged patients exhibited a much more dramatic rate of cytochrome c reduction than sera of young to middle-aged patients (Table 4, Fig. 3). Sex differences, if any, were negligible with sera from both male and female patients giving similar responses.

Table 4. Reduction of cytochrome c by sera comparing individuals aged 21-46 years and individuals aged 76-98 years and response to superoxide dismutase (SOD) and ubiquinone (Q<sub>10</sub>)

Patient age	nmoles/min/ml/sera		
	No Addition	+ 15 mg SOD	+ 45 mg Q <sub>10</sub>
21-46 years (n=16)	0.02 ± 0.1	0.02 ± 0.05	--
76-98 years (n=15)	1.5 ± 0.9	1.3 ± 0.8	--
83-95 years (n=5)	3.9 ± 1.6	--	2.5 ± 1.4

Unlike the UV-induced changes with cells (Table 3, Fig. 3), the reduction of cytochrome c by sera of aged patients occurred spontaneously (no need for UV induction) and was only partially inhibited by SOD. An interesting feature of the reduction of cytochrome c by sera of aged patients was that the activity was inhibited 36% on average by ubiquinone. The degree of inhibition by 0.1 mM ubiquinone varied from 6 to 90%.

NOX activities of cells, plasma membrane and sera (as well as the purified protein) oscillate with a period of 24 minutes. The oscillations are given by both the oxidation of NADH and by the oxidation of ubiquinol or reduced vitamin K<sub>1</sub> (Morré, 1998, Plasma Membrane Redox Systems and their Role in Biological Stress and Disease, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 121-156). The oscillations have been observed for several NADH oxidase activity forms such that the oscillations now serve in the laboratory as a diagnostic feature to identify NOX activity forms during their purification (Morré, 1998, Plasma Membrane Redox Systems and their Role in Biological Stress and Disease, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 121-156).

Sera of healthy individuals normally exhibit an NADH oxidase activity with a major period of 24 minutes (single arrows, Fig. 2A). There is, however, a second set of oscillations that conform to a period of about 26 min (double arrows, Fig. 2A). The latter oscillations are augmented in sera of aged patients (Fig. 2B). The aging-related oscillatory activity represents a unique isoform since it is resistant to inhibition by retinoic acid (10 mM) whereas the isoform with a 26 minute period in sera of healthy individuals appears to be inhibited by retinoic acid.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.



Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

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## WHAT IS CLAIMED IS:

1. A method of preventing a complication of a primary disorder in patients wherein said complication results from oxidative damage resulting from the generation of reactive oxygen species by AR-NOX, which comprises:  
administering to a patient having said primary disorder, in an amount effective to prevent said complication, one or more ubiquinones in a pharmaceutically acceptable carrier.
2. A method for preventing secondary disorders in patients having a primary disorder that causes oxidative damage resulting from the generation of reactive oxygen species by AR-NOX, which comprises:  
administering to a patient having a primary disorder, in an amount effective to prevent said secondary disorder, one or more ubiquinones in a pharmaceutically acceptable carrier.
3. The method of claim 1 or 2 wherein the total daily amount of ubiquinone administered is from about 1 to about 500 mg of a composition comprising ubiquinones.
4. The method of claim 3 wherein the total daily amount of ubiquinone administered is from about 1 to about 100 mg of the composition comprising ubiquinones.
5. The method of claim 1 or 2 wherein the ubiquinone is coenzyme Q<sub>10</sub>.
6. The method of claim 1 or 2 wherein said ubiquinone is not coenzyme Q<sub>10</sub>.
7. The method of claim 1 or 2 wherein coenzyme Q<sub>10</sub> is administered with a ubiquinone selected from a group consisting of coenzyme Q<sub>6</sub>, coenzyme Q<sub>7</sub>, coenzyme Q<sub>8</sub>, or coenzyme Q<sub>9</sub>.
8. The method of claim 1 or 2 wherein the primary disorder is old age, rheumatoid arthritis, arthritis associated with age, or fatigue associated with age.



9. The method of claim 1 or 2 wherein the primary disorder is a result of aged cells.

10. The method of claim 1 or 2 wherein the primary disorder is cancer.

5

11. The method of claim 1 or 2 wherein the primary disorder is selected from a group comprising myocardial infarction, alcoholism, favism, malaria, sickle cell anemia, Fanconi's anemia, protoporphyria photo-oxidation, nutritional deficiencies, Kwashiorkor, thalassemia, dietary iron overload, idiopathic hemochromatosis, metal ion-mediated nephrotoxicity, aminoglycoside nephrotoxicity, autoimmune nephrotic syndromes, oral iron poisoning, endotoxin liver injury, free fatty acid-induced pancreatitis, nonsteroidal antiinflammatory drug induced gastrointestinal tract lesions, glomerulonephritis, autoimmune diseases, vasculitis, hepatitis B virus, Parkinson's disease, neurotoxins, allergic encephalomyelitis, potentiation of traumatic injury, hypertensive cerebrovascular injury, vitamin E deficiency, adriamycin cardiotoxicity, Keshan disease, selenium deficiency, alcohol cardiomyopathy, photic retinopathy, ocular hemorrhage, cataractogenesis, degenerative retinal damage, amyotrophic lateral sclerosis, age-related macular degeneration, diabetes, atherogenesis, and atherosclerosis.

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12. A method for screening for agents that sequester AR-NOX, comprising:  
 (a) incubating in a reaction comprising AR-NOX and a test agent for a time sufficient to allow the test agent to bind AR-NOX; and  
 (b) detecting in the reaction the presence of a complex comprising AR-NOX and the test compound.

25

13. The method of claim 12 wherein the test agent is detectably labeled by a dye, an enzyme, an isotope, a fluorescent group, or a luminescent group.

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14. The method of claim 12 which further comprises incubating AR-NOX in the presence of a positive control that is known to interact with AR-NOX.

15. The method of claim 14 wherein the positive control is ubiquinone.

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16. The method of claim 12 wherein the method of screening takes place within a cell.

17. A method of screening for agents that sequester AR-NOX comprising:
  - (a) incubating in a reaction comprising a mixture of AR-NOX, a test agent, cytochrome c, and a substrate capable of generating reactive oxygen species for a time sufficient for cytochrome c reduction; and
  - (b) detecting the presence of reduced cytochrome c in the presence or absence of the test compound.
18. The method of claim 17 wherein the compound capable of generating reactive oxygen species is superoxide dismutase.
19. The method of claim 17 wherein the detection of cytochrome c is measured by comparing the measure of spectrophotometric absorbance of the mixture at about 540 nm to 550 nm.
20. A method of screening for agents that sequester AR-NOX comprising:
  - (a) incubating in a reaction comprising a mixture of AR-NOX, a test agent, and a substrate, wherein said substrate is reduced by AR-NOX, for a time sufficient for AR-NOX to reduce said substrate; and
  - (b) detecting the presence of reduced substrate in the presence or absence of the test compound.
21. The method of claim 20 wherein the substrate is an ascorbate radical.
22. The method of claim 21 wherein the detection of ascorbate radical is measured by comparing the measure of spectrophotometric absorbance of the mixture at about 265 nm.
23. The method of claim 20 wherein the substrate is  $\text{NAD}^+$ .
24. A method of screening for agents that sequester AR-NOX comprising:
  - (a) incubating in a reaction comprising a mixture of AR-NOX, a test agent, and a substrate, wherein said substrate undergoes disulfide-thiol interchange activity in the presence of AR-NOX, for a time sufficient for AR-NOX to reduce said substrate; and



- (b). detecting the presence of disulfide-thiol interchange in the substrate in the presence or absence of the test compound.

25. A method of preventing a complication of a primary disorder in patients  
5 wherein said complication results from oxidative damage resulting from the generation of reactive oxygen species by AR-NOX, which comprises:

administering to a patient having said primary disorder, in an amount effective to prevent said complication, an agent that sequesters AR-NOX, identified by the methods of claims 12, 17, 20, or 24, in a pharmaceutically acceptable carrier.

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26. A method for preventing secondary disorders in patients having a primary disorder that causes oxidative damage resulting from the generation of reactive oxygen species by AR-NOX, which comprises:

administering to a patient having a primary disorder, in an amount  
15 effective to prevent said secondary disorder, an agent that sequesters AR-NOX, identified by the methods of claims 12, 17, 20, or 24, in a pharmaceutically acceptable carrier.

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27. The method of claim 25 wherein the primary disorder is old age, rheumatoid arthritis, arthritis associated with age, or fatigue associated with age.

28. The method of claim 26 wherein the primary disorder is old age, rheumatoid arthritis, arthritis associated with age, or fatigue associated with age.

29. The method of claim 25 wherein the primary disorder is selected from a  
25 group comprising cancer, myocardial infarction, alcoholism, favism, malaria, sickle cell anemia, Fanconi's anemia, protoporphyria photo-oxidation, nutritional deficiencies, Kwashiorkor, thalassemia, dietary iron overload, idiopathic hemochromatosis, metal ion-mediated nephrotoxicity, aminoglycoside nephrotoxicity, autoimmune nephrotic syndromes, oral iron poisoning, endotoxin liver injury, free fatty acid-induced pancreatitis, nonsteroidal  
30 antiinflammatory drug induced gastrointestinal tract lesions, glomerulonephritis, autoimmune diseases, vasculitis, hepatitis B virus, Parkinson's disease, neurotoxins, allergic encephalomyelitis, potentiation of traumatic injury, hypertensive cerebrovascular injury, vitamin E deficiency, adriamycin cardiotoxicity, Keshan disease, selenium deficiency, alcohol cardiomyopathy, photic retinopathy, ocular hemorrhage, cataractogenesis, degenerative  
35 retinal damage, amyotrophic lateral sclerosis, age-related macular degeneration, diabetes, atherogenesis, and atherosclerosis.

30. The method of claim 26 wherein the primary disorder is selected from a group comprising cancer, myocardial infarction, alcoholism, favism, malaria, sickle cell anemia, Fanconi's anemia, protoporphyria photo-oxidation, nutritional deficiencies, Kwashiorkor, thalassemia, dietary iron overload, idiopathic hemochromatosis, metal ion-mediated nephrotoxicity, aminoglycoside nephrotoxicity, autoimmune nephrotic syndromes, oral iron poisoning, endotoxin liver injury, free fatty acid-induced pancreatitis, nonsteroidal antiinflammatory drug induced gastrointestinal tract lesions, glomerulonephritis, autoimmune diseases, vasculitis, hepatitis B virus, Parkinson's disease, neurotoxins, allergic encephalomyelitis, potentiation of traumatic injury, hypertensive cerebrovascular injury, vitamin E deficiency, adriamycin cardiotoxicity, Keshan disease, selenium deficiency, alcohol cardiomyopathy, photic retinopathy, ocular hemorrhage, cataractogenesis, degenerative renal damage, amyotrophic lateral sclerosis, age-related macular degeneration, diabetes, atherogenesis, and atherosclerosis.

31. The method of claim 25 wherein a response to the agent that sequesters AR-NOX is monitored in sera.

32. The method of claim 26 wherein a response to the agent that sequesters AR-NOX is monitored in sera.

33. The method of claim 31 wherein AR-NOX is detected with an antibody specific to AR-NOX.

34. The method of claim 32 wherein AR-NOX is detected with an antibody specific to AR-NOX.

35. The method of claim 33 wherein the antibody is conjugated to a label wherein the label provides a detectable signal.

36. The method of claim 34 wherein the antibody is conjugated to a label wherein the label provides a detectable signal.

37. The method of claim 31 wherein AR-NOX is detected by an enzymatic assay.



38. The method of claim 32 wherein AR-NOX is detected by an enzymatic assay.
39. The method of claim 37 wherein the assay is reduction of cytochrome c.
40. The method of claim 38 wherein the assay is reduction of cytochrome c.
41. The method of claim 37 wherein the assay is reduction of an ascorbate radical.
42. The method of claim 38 wherein the assay is reduction of an ascorbate radical.
43. The method of claim 37 wherein the assay is oxidation of NADH.
44. The method of claim 38 wherein the assay is oxidation of NADH.
45. The method of claim 37 wherein the assay is measuring disulfide-thiol interchange activity.
46. The method of claim 38 wherein the assay is measuring disulfide-thiol interchange activity.
47. The method of claim 31 wherein AR-NOX is detected by measuring an oscillation rate of AR-NOX.
48. The method of claim 32 wherein AR-NOX is detected by measuring an oscillation rate of AR-NOX.
49. The method of claim 31 wherein AR-NOX is detected by measuring its resistance to retinoic acid.
50. The method of claim 32 wherein AR-NOX is detected by measuring its resistance to retinoic acid.

51. The method of claim 31 wherein AR-NOX is detected by identifying cells with depressed mitochondrial functions.

52. The method of claim 32 wherein AR-NOX is detected by identifying  
5 cells with depressed mitochondrial functions.

53. A pharmaceutical composition comprising a compound that inhibits the generation of reactive oxygen species by AR-NOX in a cell that is identified by the methods of claims 12, 17, 20, or 24, and a pharmaceutical acceptable carrier.

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54. The composition of claim 53 wherein the composition is formulated as a capsule, tablet, ~~soft~~ gel, solution, suppository, injection, aerosol, or a kit.

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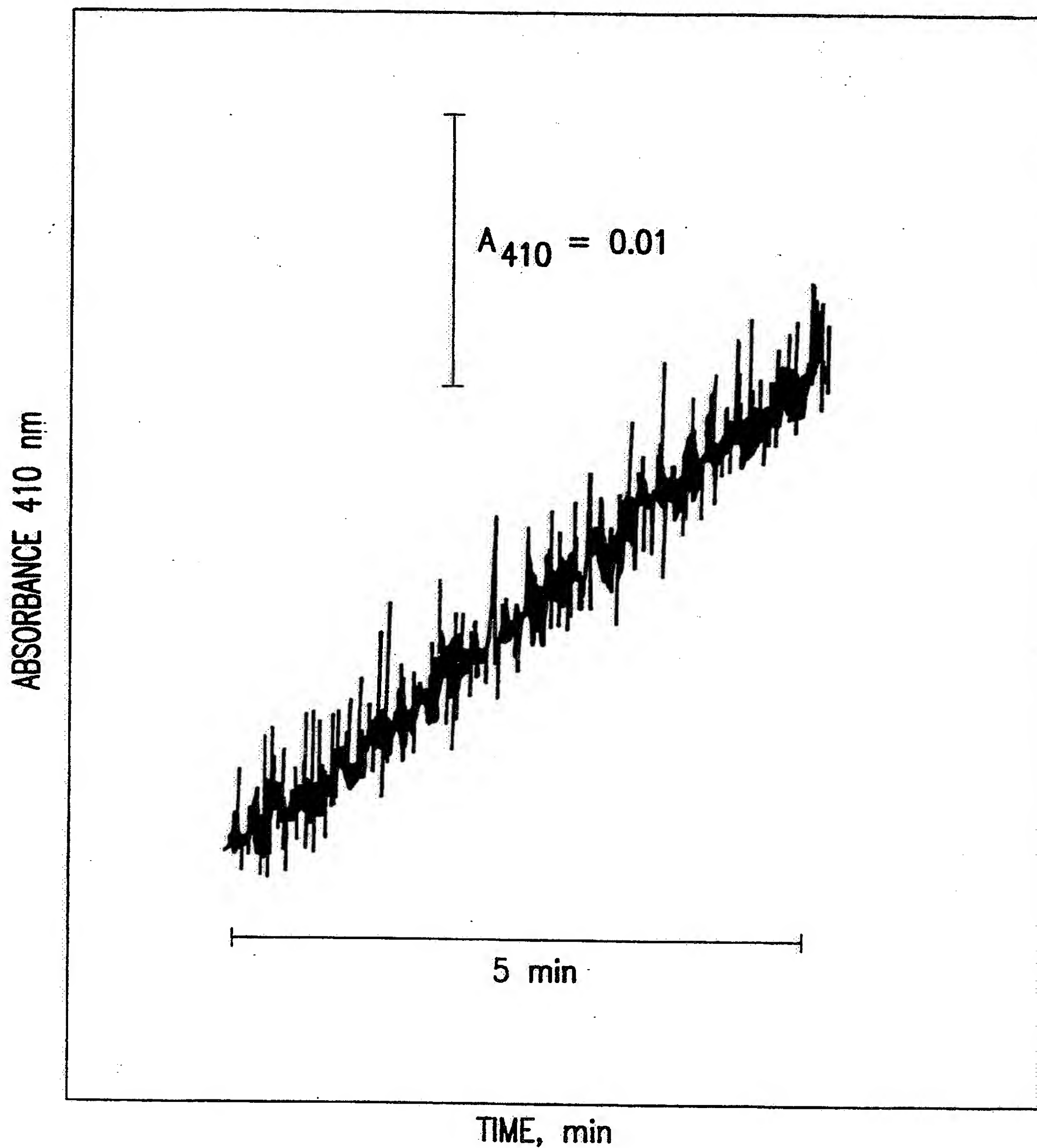


FIG.1

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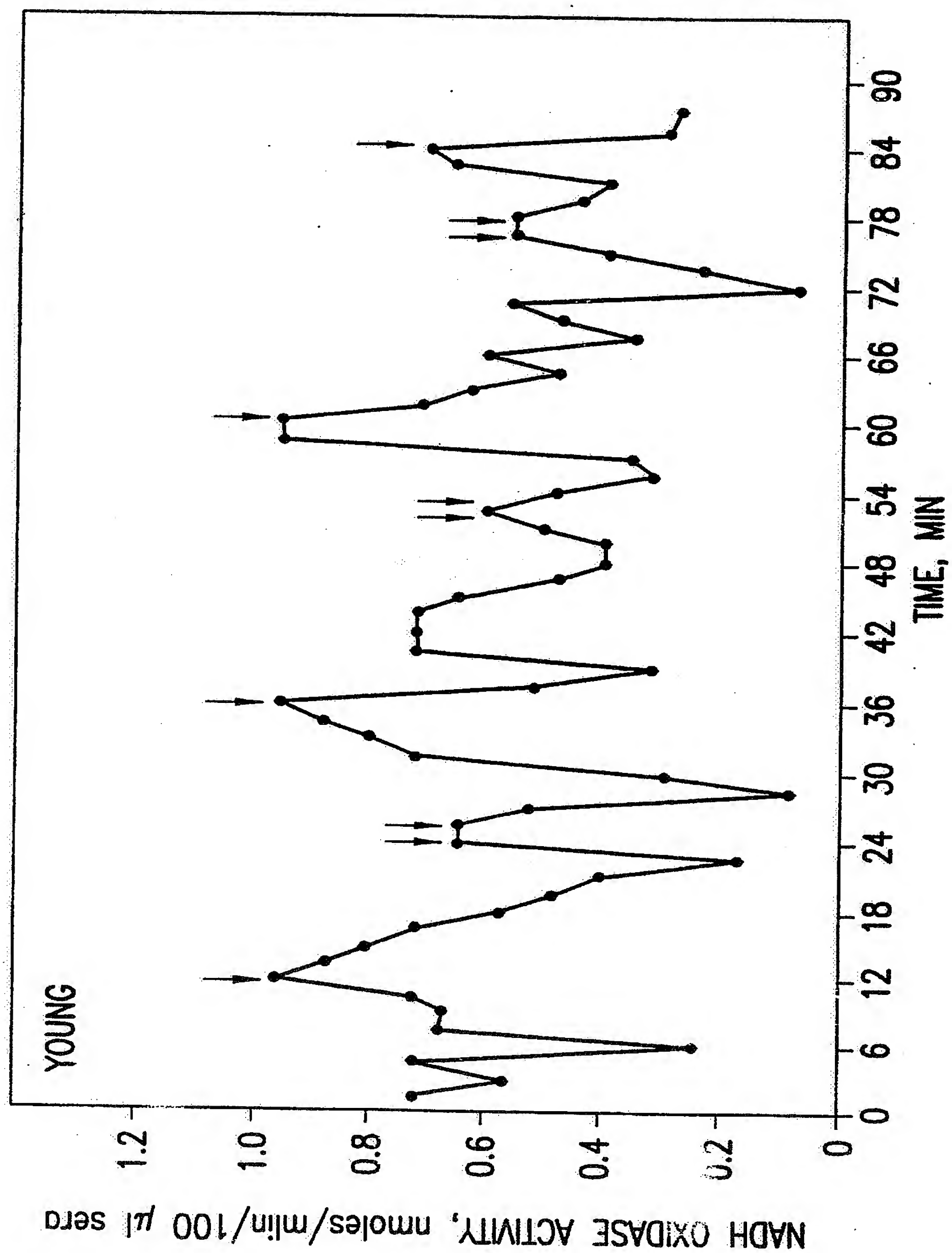


FIG. 2A



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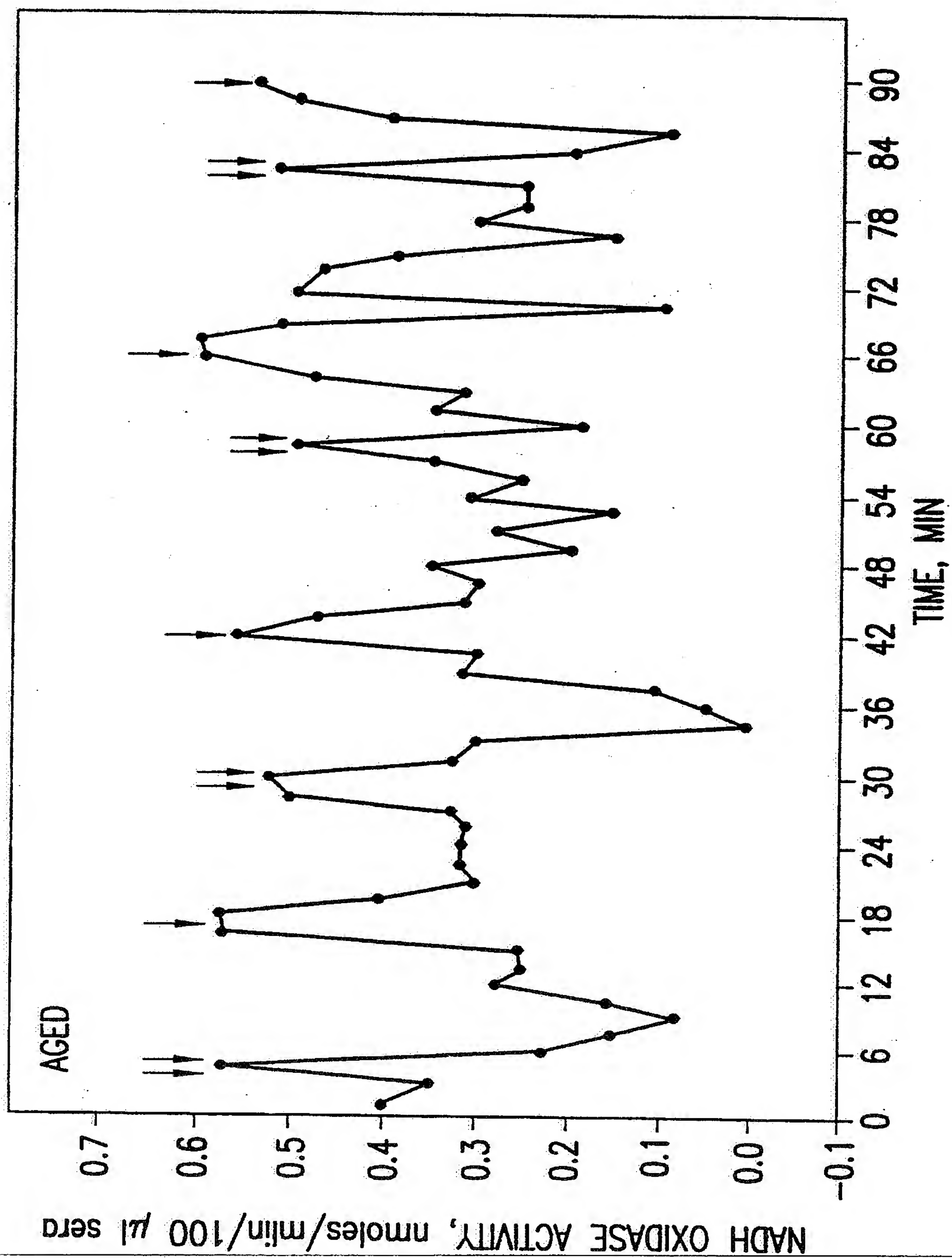


FIG. 2B

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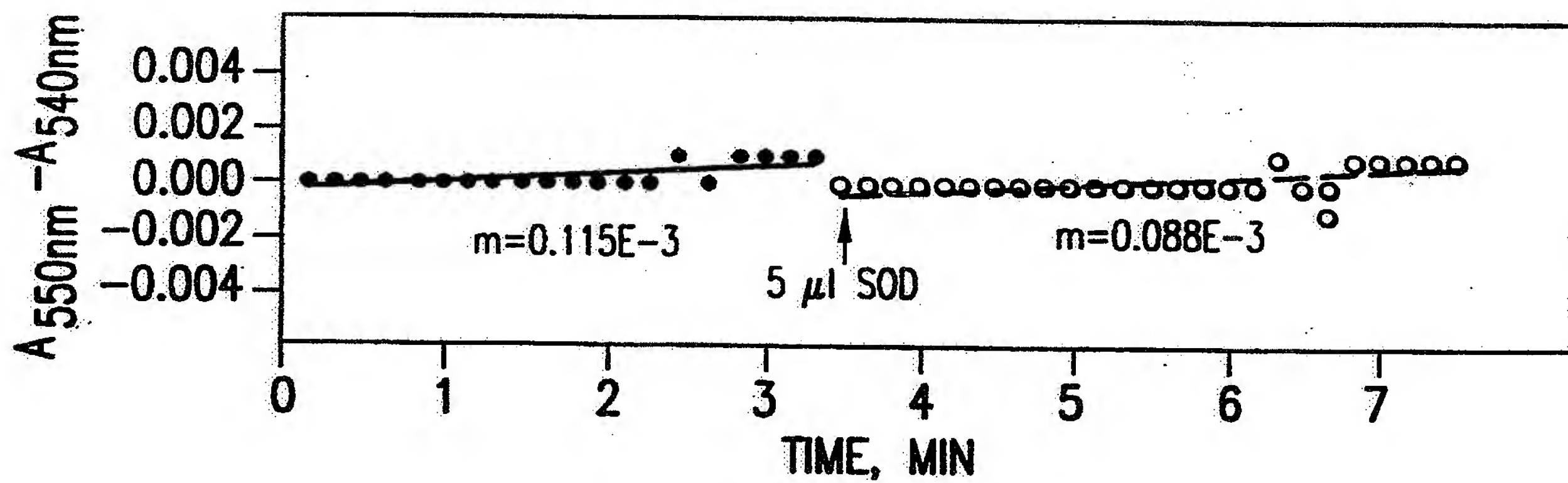


FIG. 3A

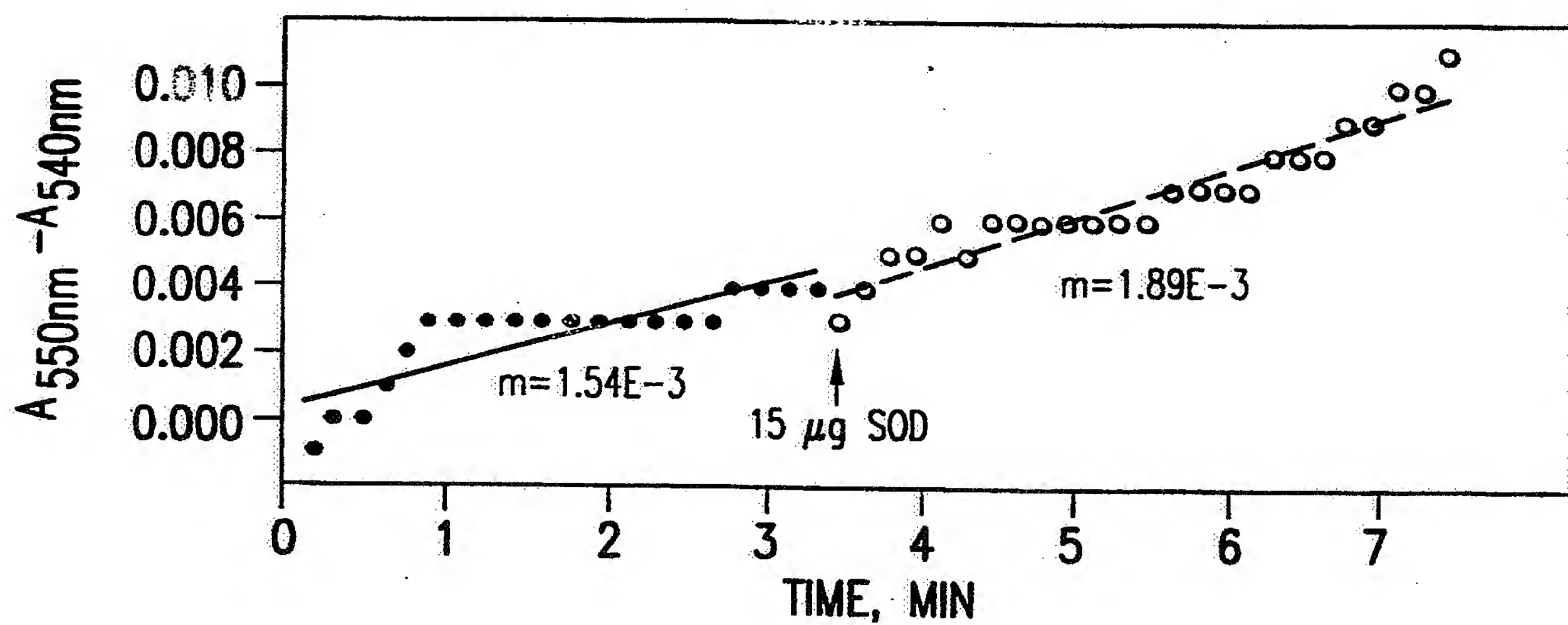
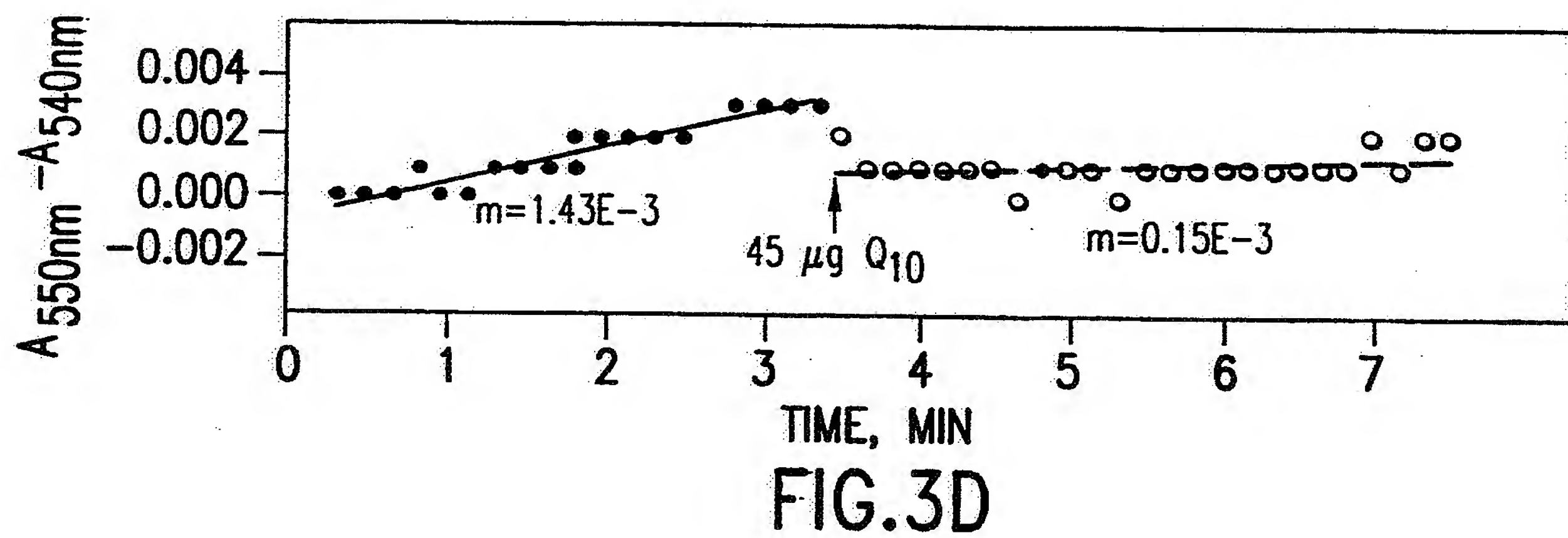
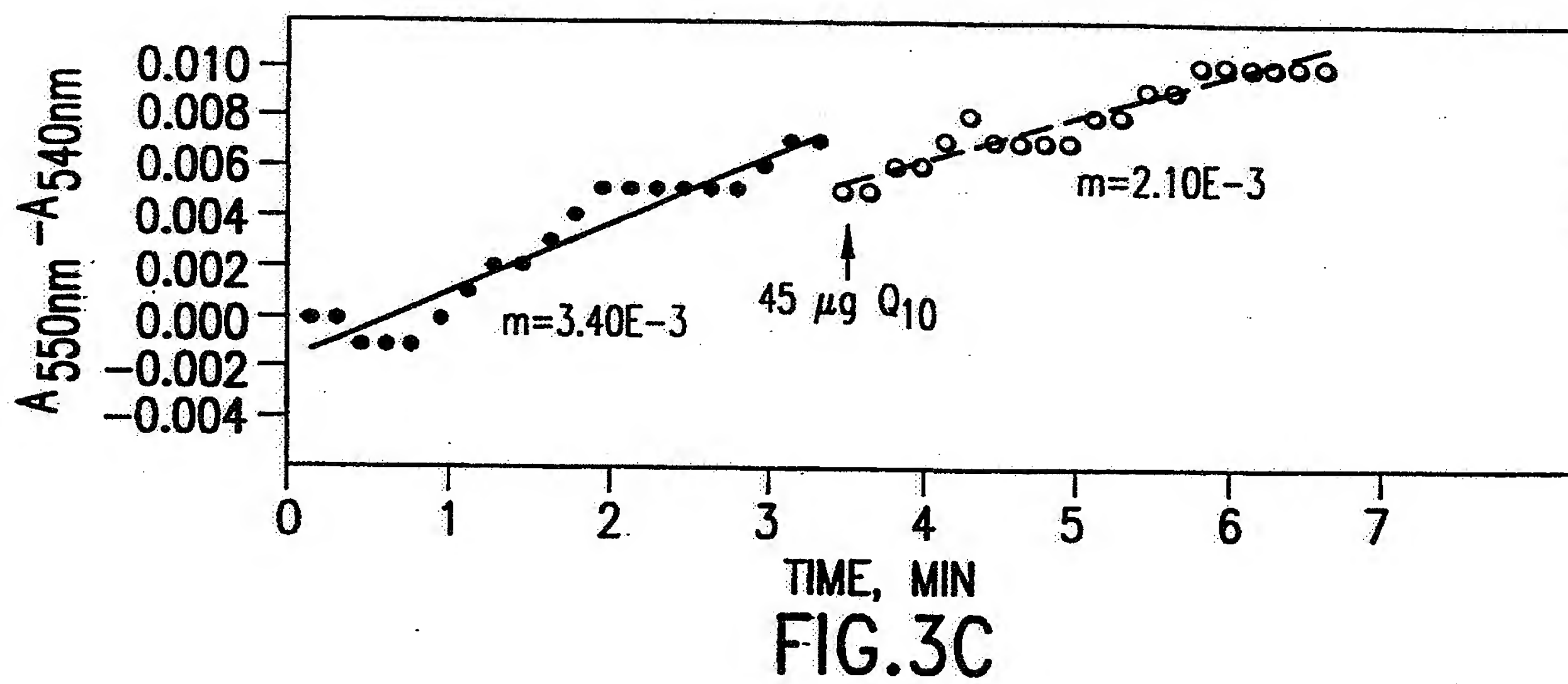


FIG. 3B

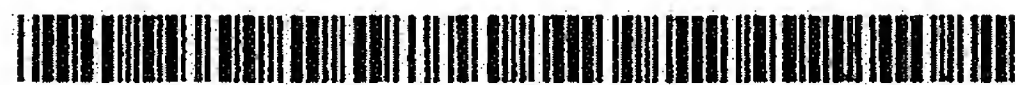


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(71) Applicant: PURDUE RESEARCH FOUNDATION [US/US]; 1063 Hovde Hall, West Lafayette, IN 47907-1063 (US).

(72) Inventors: MORRE, Dorothy, M.; 1112 Cherry Lane, West Lafayette, IN 47906 (US). MORRE, D., James; 1112 Cherry Lane, West Lafayette, IN 47906 (US).

(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— with international search report

(88) Date of publication of the international search report:  
31 January 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS FOR IDENTIFYING AGENTS THAT INHIBIT SERUM AGING FACTORS AND USES AND COMPOSITIONS THEREOF

(57) Abstract: The invention described herein encompasses methods of preventing or treating disorders caused by oxidative damage by an aging-specific isoform of NADH oxidase (AR-NOX). The invention encompasses methods of assaying, screening, and identifying agents that inhibit AR-NOX, as well as methods using ubiquinone to inhibit the ability of AR-NOX to generate reactive oxygen species. These agents may be formulated into pharmaceutical compositions in the prevention and treatment of disorders caused by oxidative damage.

WO 00/57871 A3

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 00/08433

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K31/122 A61P19/00 A61P39/06 A61K31/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, MEDLINE, EMBASE, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>NAGLEY, P. ET A.L.: "MITOCHONDRIAL DNA IN HUMAN PATHOLOGY"</p> <p>1993, RAVEN PRESS LTD., NEW YORK</p> <p>XP000982105</p> <p>page 138, paragraph 3 -page 140, paragraph 3; table 1</p> <p>page 142, paragraphs 2,3</p> <p>page 143, paragraph 3 -page 144, paragraph 2</p> <p>page 146, paragraph 3 -page 153, paragraph 2; table 3</p> <p style="text-align: center;">--- -/--</p>	1-8

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

11 July 2001

Date of mailing of the international search report

20 JULY 2001

Name and mailing address of the ISA

European Patent Office, P.B. 5618 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.  
Fax: (+31-70) 340-3016

Authorized officer

A. Jakobs



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 00/08433

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CALVANI, M. ET AL: "MITOCHONDRIAL DNA IN HUMAN PATHOLOGY" 1993, RAVEN PRESS LTD., NEW YORK XP000982106 page 173, paragraph 1 -page 175, paragraph 3 page 180, paragraph 4 -page 181, paragraph 5; tables 3,6,7	1-8
X	ROWLAND MICHAEL A ET AL: "Coenzyme Q10 treatment improves the tolerance of the senescent myocardium to pacing stress in the rat." CARDIOVASCULAR RESEARCH, vol. 40, no. 1, October 1998 (1998-10), pages 165-173, XP000978237 ISSN: 0008-6363 the whole document	1-8
X	MORTENSEN S A ET AL: "LONG-TERM COENZYME Q10 THERAPY: A MAJOR ADVANCE IN THE MANAGEMENT OF RESISTANT MYOCARDIAL FAILURE" DRUGS UNDER EXPERIMENTAL AND CLINICAL RESEARCH, BIOSCIENCE EDIPRINT INC, XX, vol. 11, no. 8, 1985, pages 581-583, XP000965535 ISSN: 0378-6501 abstract page 592, column 1, paragraph 3 -column 2, paragraph 2	1-8
X	KAMIKAWA T ET AL: "EFFECTS OF COENZYME Q-10 ON EXERCISE TOLERANCE IN CHRONIC STABLE ANGINA PECTORIS" AMERICAN JOURNAL OF CARDIOLOGY, vol. 56, no. 4, 1985, pages 247-251, XP000978238 ISSN: 0002-9149 abstract page 247, column 1, paragraph 1 -column 2, paragraph 1; figure 3; tables 3,4 page 250, column 1, paragraph 3 -column 2, paragraph 4	1-8
X	FR 2 763 850 A (SLOW AGE CREATIONS) 4 December 1998 (1998-12-04) abstract page 3, line 1-14 claims 1-13; examples 1-3	1-8
X	WO 98 35658 A (ENZMANN FRANZ ;MSE PHARMAZEUTIKA GMBH (DE); LACHMANN BURKHARD (NL)) 20 August 1998 (1998-08-20) claims 1-6	1

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 00/08433

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 056 613 A (BERTAZZOLI CESARE ET AL) 1 November 1977 (1977-11-01) abstract; claim 1	1
X	LENAZ GIORGIO ET AL: "Oxidative stress, antioxidant defences and aging." BIOFACTORS, vol. 8, no. 3-4, 1998, pages 195-204, XP000979637 ISSN: 0951-6433 the whole document	1-8
A	SHERRATT E J ET AL: "MITOCHONDRIAL DNA DEFECTS: A WIDENING CLINICAL SPECTRUM OF DISORDERS" CLINICAL SCIENCE, BIOCHEMICAL SOCIETY AND THE MEDICAL RESEARCH SOCIETY, LONDON., GB, vol. 92, March 1997 (1997-03), pages 225-235, XP000943576 ISSN: 0143-5221 the whole document	1-11
A	GENOVA MARIA LUISA ET AL: "Decrease of rotenone inhibition is a sensitive parameter of complex I damage in brain non-synaptic mitochondria of aged rats." FEBS LETTERS, vol. 410, no. 2-3, 1997, pages 467-469, XP000979254 ISSN: 0014-5793 the whole document	1
A	BERKOW R ET AL: "MERCK MANUAL OF DIAGNOSIS AND THERAPY" RAHWAY, MERCK & CO, US, 1987, page 2392 XP002141064 the whole document	1-12
A	HILDEBRANDT H (ED ): "PSCHYREMBEL KLINISCHES WOERTERBUCH" BERLIN: WALTER DE GRUYTER, DE, vol. ED. 258, 1998, pages 47-49, XP002141063 ISBN: 3-11-015676-8 the whole document	1-12
A	BEERS M H ET AL "MERCK MANUAL OF DIAGNOSIS AND THERAPY" CENTENNIAL EDITION, WHITEHOUSE STATION, NJ: MERCK RES. LAB, US, 1999, pages 2503-2506, XP002141065 ISBN: 0-911910-10-7 the whole document	1-12

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# INTERNATIONAL SEARCH REPORT

Inte. .onal Application No

PCT/US 00/08433

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PORTER T H ET AL: "SYNTHESIS ENZYME INHIBITION AND ANTI TUMOR ACTIVITY OF NEW 1 4 BENZO QUINONE ANALOGS OF COENZYME Q-10"</p> <p>BIOORGANIC CHEMISTRY, vol. 7, no. 3, 1978, pages 333-350, XP001011285 EN ISSN: 0045-2068 abstract page 338, paragraph 3 -page 347, paragraph 1; figure 1; tables 3-5</p>	<p>1-7,10, 26,29, 30,53,54</p>
X	<p>COLES, L. STEPHEN ET AL: "Coenzyme Q-10 and lifespan extension"</p> <p>ADV. ANTI-AGING MED. (1996), 205-215. EDITOR(S): KLATZ, RONALD M. PUBLISHER: LIEBERT, LARCHMONT, N. Y. , 1996, XP001011292 the whole document</p>	<p>1-8,10, 26,29, 30,53,54</p>
X	<p>LOCKWOOD, KNUD ET AL: "Progress on therapy of breast cancer with vitamin Q10 and the regression of metastases"</p> <p>BIOCHEM. BIOPHYS. RES. COMMUN. (1995), 212(1), 172-7 , 1995, XP001009781 abstract</p>	<p>1-7,10, 26,29, 30,53,54</p>
X	<p>IWASA H ET AL: "EFFECT OF COENZYME Q-10 AND COMBINED THERAPY OF COENZYME Q-10 AND FT-207 FTORAFUR FOR MICE BEARING METHA TUMOR 2"</p> <p>JOURNAL OF THE NATIONAL DEFENSE MEDICAL COLLEGE, vol. 7, no. 4, 1982, pages 332-339. XP001011283 ISSN: 0385-1796 abstract</p>	<p>1-7,10, 26,29, 30,53,54</p>
X	<p>LOCKWOOD K ET AL: "Apparent partial remission of breast cancer in 'high risk' patients supplemented with nutritional antioxidants, essential fatty acids and coenzyme Q10"</p> <p>MOLECULAR ASPECTS OF MEDICINE,GB,PERGAMON PRESS, OXFORD, vol. 15, no. SUPPL. 1994, pages S231-S240, XP002108766 ISSN: 0098-2997 abstract</p>	<p>1-7,10, 26,29, 30,53.54</p>
	<p>---</p> <p>-/--</p>	



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/08433

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 33495 A (CARBONE SANTO ;PASSI SIRO (IT); GUARNIERI DECIMO (IT); IDI FARMACE) 6 August 1998 (1998-08-06) abstract page 2, line 6 -page 3, line 34; claims 1-7; examples 1-4 ---	1-7,10, 26,29, 30,53,54
X	OGURA R ET AL: "ANTI OXIDATIVE EFFECTS OF VITAMIN B-6 BUTYRATE ON THE CARDIAC MITOCHONDRIAL DISORDERS INDUCED BY ADRIAMYCIN" JOURNAL OF NUTRITIONAL SCIENCE AND VITAMINOLOGY, vol. 28, no. 4, 1982, pages 329-334, XP001011282 ISSN: 0301-4800 abstract ---	1-7,10, 26,29, 30,53,54
X	HATA H: "IMMUNOLOGICAL RESPONSIVENESS OF TUMOR BEARING HOSTS 1. EFFECTS OF COENZYME Q-10 ON 20 METHYL CHOLANTHRENE CARCINOGENESIS" JOURNAL OF THE KANSAI MEDICAL UNIVERSITY, vol. 30, no. 1, 1981, pages 59-72, XP001011284 ISSN: 0022-8400 abstract ---	1-7,10, 26,29, 30,53,54
X	FOLKERS, KARL: "Relevance of the biosynthesis of coenzyme Q10 and of the four bases of DNA as a rationale for the molecular causes of cancer and a therapy" BIOCHEM. BIOPHYS. RES. COMMUN. (1996), 224(2), 358-361, 1996, XP001009783 the whole document ---	1-7,10, 26,29, 30,53,54
X	JOLLIET P ET AL: "Plasma coenzyme Q10 concentrations in breast cancer: Prognosis and therapeutic consequences." INTERNATIONAL JOURNAL OF CLINICAL PHARMACOLOGY AND THERAPEUTICS, vol. 36, no. 9, 1998, pages 506-509, XP001011227 ISSN: 0946-1965 abstract --- -/--	1-7,10, 26,29, 30,53,54

# INTERNATIONAL SEARCH REPORT

**Internal Application No**

PCT/US 00/08433

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>SUZUKI H ET AL: "EFFECTS OF IMMUNOSTIMULATION WITH OK-432 COENZYME Q-10 OR LEVAMISOLE ON DIMETHYLHYDRAZINE-INDUCED COLONIC CARCINOGENESIS IN RATS" JAPANESE JOURNAL OF SURGERY, vol. 16, no. 2, 1986, pages 152-155, XP001011254  ISSN: 0047-1909  abstract; tables 1,2  page 154, column 2, paragraph 2 -page 155, column 1, paragraph 1</p> <p>----</p>	<p>1-7, 10,  26, 29,  30, 53, 54</p>
X	<p>WO 98 35660 A (ENZMANN FRANZ ;MSE PHARMAZEUTIKA GMBH (DE); LACHMANN BURKHARD (NL)) 20 August 1998 (1998-08-20)  abstract  page 1, paragraph 1 -page 3, paragraph 1;  claims 1-3,5</p> <p>-----</p>	<p>1-7, 10,  26, 29,  30, 53, 54</p>

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 00/08433

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 1-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition as far as comprised within the first invention.
2. ☒ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:  
  
1-8, 10, 26, 29, 30, 53, 54
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.



## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-9 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is old age or fatigue associated with old age.

2. Claims: 1-9 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is rheumatoid arthritis or age related arthritis.

3. Claims: 1-7, (partially) 10

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is cancer.

4. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is selected from a group comprising myocardial infarction, adriamycin cardiotoxicity, Keshan disease.

5. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is selected from a group comprising alcoholism and alcohol cardiomyopathy.

6. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is selected from a group comprising favism, sickle cell anemia, Fanconi's anemia, thalassemia.

7. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is malaria.

8. Claims: 1-7,11 (partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is protoporphyrin photo-oxydation.

9. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is selected from a group comprising nutritional deficiency, Kwashiorkor, vitamin E deficiency, selenium deficiency.

10. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is selected from a group comprising dietary iron overload, oral iron poisoning, idiopathic hemochromatosis.

11. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is selected from a group comprising metal ion-mediated nephrotoxicities, aminoglycoside nephrotoxicities, autoimmune nephrotic syndrome, glomerulonephritis.

12. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is endotoxin liver injury.

13. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is free fatty acid pancreatitis.

14. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder are NSAID induced induced gastrointestinal lesions.

15. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is a

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

autoimmune disorder as far as not comprised within a preceeding or following invention.

## 16. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is selected from a group comprising vasculitis, atherogenesis, atherosclerosis.

## 17. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is hepatitis B.

## 18. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is selected from a group comprising Parkinson's disease, neurotoxins, allergic encephalomyelitis, hypertensive cerebrovascular injury, amyotrophic lateral sclerosis.

## 19. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is selected from a group comprising photic retinopathy, ocular hemorrhage, cataractogenesis, degenerative retinal damage, age-related macular degeneration.

## 20. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is diabetes.

## 21. Claims: 1-7,11 (partially)

Use of coenzyme Q10 for preventing a complication of a primary disorder wherein said primary disorder is potentiation of traumatic injury.

## 22. Claims: 12-54

A method for screening for agents that sequester Age-related isoforms of NADH oxidase (AR-NOX), pharmaceutical compositions comprising the compounds identified by said



FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

screening method (sic ).

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. .onal Application No

PCT/US 00/08433

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
FR 2763850	A	04-12-1998	NONE	
WO 9835658	A	20-08-1998	EP 1007018 A US 6228891 B	14-06-2000 08-05-2001
US 4056613	A	01-11-1977	AU 508282 B AU 1634476 A BE 844711 A DE 2633984 A JP 52018829 A ZA 7604565 A	13-03-1980 02-02-1978 31-01-1977 17-02-1977 12-02-1977 27-07-1977
WO 9833495	A	06-08-1998	IT RM970045 A AU 5878098 A EP 0966275 A	31-07-1998 25-08-1998 29-12-1999
WO 9835660	A	20-08-1998	AT 201988 T EP 1007021 A	15-06-2001 14-06-2000